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(54) Title: A METHOD FOR THE MODULATION OF ANGIOGENESIS

(57) Abstract: The present invention concerns a method for modulation (increase or decrease) of angiogenesis by the contact of the blood vessels with compounds derived from specific regions of various kinases.

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A METHOD FOR THE MODULATION OF ANGIOGENESIS

FIELD OF THE INVENTION

The present invention concerns a method for the regulation (increase or decrease) of angiogenesis.

5 BACKGROUND OF THE INVENTION

The term "*angiogenesis*" (also referred to at times as "*neovascularization*") is a general term used to denote the growth of new blood vessels both in normal and pathological conditions.

Angiogenesis is an important natural process that occurs during embryogenesis,
10 and in the adult healthy body in the process of wound healing, and in restoration of blood flow back into injured tissues. In females, angiogenesis also occurs during the monthly reproductive cycle to build up the uterus lining and to support maturation of oocytes during ovulation, and in pregnancy when the placenta is formed, in the process of the establishment of circulation between the mother and the fetus. The healthy body controls
15 angiogenesis through the interactions of angiogenesis-stimulating growth factors, and angiogenesis inhibitors, and the balance between the two determines whether angiogenesis is turned "on" or "off".

In the therapeutic field, there has been in recent years a growing interest in the control of angiogenesis. By one aspect, the aim was to control or diminish excessive and
20 pathological angiogenesis that occurs in diseases such as cancer, diabetic blindness, age related macular degeneration, rheumatoid arthritis, psoriasis, and some additional 70 conditions. In these pathological conditions the new blood vessels feed the diseased tissue, for example the tumor tissue, providing it with essential oxygen and nutrients thus enabling its pathological growth. In addition the pathological angiogenesis many times
25 destroys the normal tissue. Furthermore, the new blood vessels, formed for example in the tumor tissue, enable the tumor cells to escape into the circulation and metastasize in other organs. Typically, excessive angiogenesis occurs when diseased cells produce abnormal

amounts of angiogenetic growth factors, overwhelming the effect of the natural angiogenesis inhibitors present in the body.

Anti-angiogenetic therapies developed today, are aimed at preventing new blood vessel growth through the targeting and neutralization of any of the stimulators that encourage the formation of new blood vessels.

A contrasting indication of regulating angiogenesis is the stimulation of production of neovascularization in conditions where insufficient angiogenesis occurs. Typically, these conditions are diseases such as coronary artery diseases, stroke, and delayed wound healing (for example in ulcer lesions). In these conditions, when adequate blood vessels growth and circulation is not properly restored, there is a risk for tissue death due to insufficient blood flow. Typically, insufficient angiogenesis occurs when the tissues do not produce adequate amounts of angiogenetic growth-factors, and therapeutic angiogenesis is aimed at stimulating new blood vessels' growth by the use of growth factors or their mimics.

The main goal of the angiogenesis therapy is to produce a biobypass – i.e., to physically bypass diseased or blocked arteries, by tricking the body into building new blood vessels.

Tie 2 is a receptor tyrosine-kinase for the endothelium growth factor angiopoietin 1&2. Tie-2 has been shown to be involved in tumor related angiogenesis. Disruption of Tie-2 function in transgenic mice resulted in embryonic lethality secondary to characteristic vascular defects; similar defects occurred after disruption of the Tie-2 ligand. These findings indicate that the Tie2/Tie2 ligand pathway plays important roles during development of the embryonic vasculature. Thus inhibition of this pathway, for example by a soluble fragments of the Tie-2 receptor has been aimed as a strategy for the inhibition of angiogenesis.

The eukaryotic protein kinase super family is composed of enzymes that use the gamma phosphate of ATP or GTP to specifically phosphorylate serine, threonine or tyrosine residues of intracellular proteins. Many of the kinases are involved in signal transduction in multicellular organisms in a variety of cellular events. Kinases were found

to be involved in a wide range of physiological phenomena including: cellular proliferation, cellular differentiation, ontogenesis, immune response and inflammatory responses.

Undesired kinase activity has been found to be involved in proliferation-based diseases such as cancer; in non-malignant proliferative diseases such as arteriosclerosis and psoriasis; and in inflammatory responses such as septic shock. Malfunction of kinase activity has also been found to be involved in metabolic pathologies manifested by the decrease in the activity of insulin receptor kinase, such as in various types of diabetes. Agents that can modulate (by increasing or decreasing) the activity of protein kinases have a potential for the treatment of a wide variety of diseases and disorders including: cancer, autoimmune diseases, inflammation, injury, metabolic disorders, genetic disorders and the like.

PKs are known to have homologous "*kinase domains*" or "*catalytic domains*" which are responsible for the phosphorylation activity. Based on a comparison of a large number of protein kinases, it is now known that the kinase domain of protein kinases can be divided into twelve subdomains. These are regions that are generally uninterrupted by large amino acid insertions and contain characteristic patterns of conserved residues (Hanks and Hunter, "The Eukaryotic Protein Kinase Superfamily", in Hardie and Hanks ed., The Protein Kinase Facts Book, Volume I, Academic Press, Chapter 2, 1995). These subdomains are referred to as Subdomain I through Subdomain XII.

Due to the high degree of homology found in the subdomains of different protein kinases, the amino acid sequences of the domains of different PKs can be aligned. Frequently, the alignment is with reference to the prototypical protein kinase PKA-C α , as known in the art. Currently, the catalytic domains of a large number of protein kinases have been aligned and tables showing these alignments are available from various published sources, such as, for example, in the article by Hanks and Quinn in Methods of Enzymology 200, 38-62 (1991) or in the PKR Web Site: WWW.sdsc.edu/kinases.

US patent 6,174,993, WO 98/53051 (corresponding to pending US application 08/861,153), WO 00/118895 (corresponding to pending US 09/161,094), US pending

application 09/458,491 and US 09/734,520 (all incorporated herein by reference) concern small, previously undisclosed, regions of various protein kinases with a high substrate specificity. Short peptides derived from these regions modulate kinase activities, as determined by the modulation of cellular activity in various *in vivo* and *in vitro* models.

5 Without wishing to be bound by theory it is assumed that the short peptides disclosed in these applications, which mimic some of the catalytic domains of the kinase, bind to other cellular components with which the kinase interacted (such as substrates, other kinases, other phosphatases, co-factors, effectors, etc.) and thus either mimic the kinase activity, or alternatively inhibits the interaction of the kinase and the cellular components thus
10 inhibiting kinase activity.

US 6,174,993 and US application 08/861,153 discloses a domain termed the HJ-loop. The "HJ-loop" referred to herein is found within the kinase domain of protein kinases between the middle of Subdomain IX and the middle of Subdomain X. Because of the high degree of homology found in the subdomains of different protein kinases, the
15 amino acid sequences of the domains of different protein kinases can be aligned. Thus, the HJ-loop of protein kinases can be defined by reference to the amino acid sequence of a prototypical protein kinase, for example PKA-C α , and can be said to correspond to a contiguous sequence of about twenty amino acid residues found between about amino acid 229 and 248 of PKA-C α .

20 A second definition of the HJ loop of protein kinases, which is complementary to the definition provided in the preceding paragraph, can be made by reference to the secondary structure of the kinase domain of protein kinases. The kinase domain of protein kinases has been found to contain at least nine alpha helices, referred to as helix A through helix I, nine beta sheets, referred to as b1 through b9 (Tabor *et al.*, *Phil. Trans. R. Soc. Lond.* **B340**:315 (1993), Mohammadi *et al.*, *Cell* **86**:577 (1996) and Hubbard *et al.*,
25 *Nature* **372**:746 (1994)). The HJ loop is a contiguous sequence of about twenty amino acids beginning within the F helix about five amino acids residues from the N-terminus of the F helix and extending about five amino acid residues into the G helix.

US patent application 09/734,520 discloses a region termed the "*A-region*". The "*A region*" referred to herein is found within the kinase domain of PKs in Subdomain III and Subdomain IV. With respect to the amino acid sequence of the prototypical protein kinase PKA-C α the A region can be said to correspond to a contiguous sequence of about
5 eighteen amino acid residues found between about amino acids 92 and 109 of PKA-C α . In some PKs, extra amino acids can be present in this region and the size of the A region can, therefore, include more than 18 amino acids in length.

With respect to the secondary structure of protein kinases, the A region is a contiguous sequence of about five to twenty amino acids beginning at the middle of the
10 α C helix (hereby α C) and ending at the beginning of the b4 beta sheet.

US patent application 09/458,491 discloses a region termed B4-B5 region. The "*B4-5 region*" referred to herein is found within the kinase domain of PKs in Subdomain IV and the beginning of Subdomain V. With respect to the amino acid sequence of the prototypical protein kinase PKA-C α , the B4-5 region can be said to correspond to a
15 contiguous sequence representing the amino acid residues found between about amino acids 106 and 114 of PKA-C α .

In some PKs, extra amino acids might be inserted in this region and the size of the B4-5 region can, therefore, include more than 9 amino acids in length.

A second definition of the B4-5 region of a PK, which is complementary to the
20 definition provided in the preceding paragraph, can be made by reference to the three dimensional structure of the kinase domain of PKs. The kinase domain of PKs has been found to contain at least nine alpha helices, referred to as helix A through helix I and nine beta sheets, referred to as b1 through b9 (Tabor *et al.*, *Phil. Trans. R. Soc. Lond.*, **B340**:315 (1993), Mohammadi *et al.*, *Cell*, **86**:577 (1996) and Hubbard *et al.*, *Nature*
25 **372**:746 (1994). The B4-5 region is a contiguous sequence of about five to twenty five amino acids beginning at the end of the b4 beta sheet and into the b5 beta sheet.

US patent application 09/161,095 discloses a region termed the " *α D region*". The " *α D region*" referred to herein is found within the kinase domain of PKs in Subdomain V and the beginning of Subdomain VI. The " *α D region*" of a PK can be defined by

reference to the amino acid sequence of a prototypical protein kinase, for example PKA-C α and can be said to correspond to a contiguous sequence of about twenty amino acid residues found between about amino acid 120 and 139 of PKA-C α .

In relation to the secondary structure of the kinase domain of PKs, the α D region is
5 a contiguous sequence of about fifteen to forty amino acids beginning at the end of the b5 beta sheet and extending through the D helix and the following loop to the beginning of helix E.

The three dimensional structure of a number of kinases has been determined. A classical view of this structure is given in Knighton *et al.*, *Science* **253**, 407-414 (1991).

10 SUMMARY OF THE INVENTION

The present invention is based on the finding that compounds comprising peptides, which are derived from specific regions of Tie-2, c-kit, ILK, VEGF and GRK kinases, or variants of these peptides, were able to inhibit or stimulate angiogenesis in several different model of angiogenesis both *in vivo* and *in vitro*.

15 Without wishing to be bound by theory, it is assumed that the peptide part of the compound, mimics a region of the Tie-2, c-kit, ILK, VEGF and GRK kinases, which region is responsible for interactions with other cellular components such as: the substrates of the kinase; phosphatases or other kinases which regulate the level of phosphorylation of these kinases, co-factors, ATP and the like. The peptide part of the compound thus
20 interrupts the interaction of the native Tie-2, c-kit, ILK, VEGF and GRK kinases with the other cellular components (for example the interactions of the kinase with the substrates of this kinase) and this interruption modulates (increases or decreases) the signal-transduction associated by the Tie-2, c-kit, ILK, and GRK kinases.

Thus the present invention concerns a method for the modulation of angiogenesis
25 comprising contacting blood vessels with an effective amount of a compound comprising a sequence selected from:

- (a) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 1030 to 1052, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions

861 to 885; or a continuous stretch of at least five amino acids present in a native ILK in positions of 382 to 407; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 1097 to 1121; continuous stretch of at least five amino acids present in a native GRK in positions of 383 to 406 (all denoted HJ-loop);

(b) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 904 to 923, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 672 to 694; or a continuous stretch of at least five amino acids present in a native ILK in positions of 271 to 290; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 918 to 939; continuous stretch of at least five amino acids present in a native GRK in positions of 271 to 291(all denoted α D region);

(c) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 887 to 903, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 655 to 671; or a continuous stretch of at least five amino acids present in a native ILK in positions of 256 to 269; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 900 to 917; continuous stretch of at least five amino acids present in a native GRK in positions of 257 to 271(all denoted B4-B5 region);

(d) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 874 to 891, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 640 to 668; or a continuous stretch of at least five amino acids present in a native ILK in positions of 235 to 258; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 884 to 903; continuous stretch of at least five amino acids present in a native GRK in positions of 239 to 260 (all denoted A-region);

- (e) a variant of a sequence according to any one of (a) to (d) wherein up to 40% of the amino acid of the native sequence have been replaced with a naturally or non-naturally occurring amino acid or with a peptidomimetic organic moiety; and/or up to 40% of the amino acids have their side chains chemically modified; and/or up to 20% of the amino acids have been deleted; provided that at least 50% of the amino acids in the parent sequence of (a) to (d) are maintained unaltered in the variant, and provided that the variant has autoimmune disease improving properties;
- (f) a sequence of any one of (a) to (e) wherein at least one of the amino acids is replaced by the corresponding D- amino acid;
- (g) a sequence of any one of (a) to (f) wherein at least one of the peptidic backbones has been altered to a non-naturally occurring peptidic backbone;
- (h) a sequence being the sequence of any one of (a) to (g) in reverse order; and
- (i) a combination of two or more of the sequences of (a) to (h).

Preferably the sequences from C-KIT, VEGF and Tie-2 are from the alpha D region (b) above and its derivatives) and sequences from GRK or ILK are from the HJ-region and its derivatives ((a) above). Where the sequence is derived from alpha D region of c-KIT the subsequence of (b) is from positions 677-689; and where the sequence is derived from the alpha D region of Tie2 the subsequence is from positions 909 to 920

By a most preferred aspect, the above method of regulating angiogenesis-controlling genes may be used for therapeutical purposes, i.e., to prevent, treat, or alleviate at least one of the undesired systems of diseases, disorders or pathological conditions which are manifested by non-normal (over or under) angiogenesis.

By one aspect, termed the "*pro-angiogenesis aspect*" the therapeutical methods is used to increase angiogenesis-, for the purpose of prevention, treatment or alleviation of diseases or conditions wherein a beneficial therapeutical affect may be evident by neovascularization.

Examples of such diseases are coronary artery diseases, peripheral artery diseases, endothelial vascular diseases, arteriosclerosis, various processes of wound and tissue healing such as healing of bone, tendon, endothelial lining (such as in ulcers in the stomach or the skin), for improving the success rates of cell transplantation techniques, as well as in reconstructive surgery to help re-establish proper blood circulation to the reconstructed tissue.

According to the pro-angiogenesis aspect the sequence as defined in (a) to (i) above is derived from ILK, and in particular from the HJ-region of ILK (a) above) or a derivative of such a sequence (e) to (i) above.

10 By another aspect termed "*anti-angiogenesis aspect*" the method of the present invention may be used for the treatment, prevention or alleviation of diseases, conditions and disorders wherein a therapeutical beneficial affect is evident through the inhibition, decrease or the prevention of angiogenesis.

Examples of such diseases are cancer, aged-related macular degeneration
15 (which are many times aggravated by normal neovascularization), diabetic retinopathy (which are also caused by non normal neovascularization), rheumatoid arthritis, psoriasis, obesity, hemangioma (AIDS related), Kaposi sarcoma, arteriosclerosis, and restenosis.

In particular the anti-angiogenesis aspect is used for the treatment of solid
20 tumors such as for example: carcinoma, sarcoma, adenoma, hepatocellular carcinoma, hepatocellular carcinoma, hepatoblastoma, rhabdomyosarcoma, esophageal carcinoma, thyroid carcinoma, ganglioblastoma, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, synovioma, Ewing's tumor, leiomyosarcoma,
25 rhabdotheliosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, renal cell carcinoma, hematoma, bile duct carcinoma, melanoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small lung carcinoma, bladder carcinoma, epithelial

carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, retinoblastoma,, multiple myeloma, rectal carcinoma, thyroid cancer, head and neck cancer, brain cancer, cancer of the peripheral nervous system, cancer of the central nervous system, neuroblastoma, cancer of the endometrium, as well as
5 metastasis of all the above

According to the anti-angiogenesis aspect the sequence part of the compound is derived from the Tie-2, VEGF or c-kit kinases (and in particular the alpha D region of these kinases or derivatives of that region as defined in (e) to (i) above), the GRK kinase (in particular the HJ-region of the kinase or derivatives of hat sequence as
10 defined in (e) to (i) above.

The term "*modulating*" in the context of the "*pro-angiogenetic*" aspect of the present invention concerns increase in the formation and/or growth rate of new blood vessels, stabilization and/or elongation of existing blood vessels.

This term concerns in the "*anti-angiogenetic aspect*" of the present invention
15 decrease in the formation and/or growth rate of new blood vessels, destabilization and/or retraction of existing blood vessels. The increase/decrease may be only in the presence of the compound of the invention as compared to the angiogenesis in the absence of the compound of the invention (for example in the presence of a control compound). Alternatively the increase/decrease may be a change in the response to an external signal
20 such as stress, presence of a growth factor etc., in the presence of the compound of the invention as compared to the response to the same external signal in the absences of the compound of the invention.

The term "*compound (comprising sequence)*" refers to a compound that includes within any of the sequences of (a) to (i) as defined above. The compound may be
25 composed mainly from amino acid residues, and in that case the amino acid component of the compound should comprise no more than a total of about 55 amino acids. Where the compound is mainly an amino acid compound, it may consist of any one of the amino acid sequences of (a) to (h), a combination of two or more, preferably of three most preferably of two, of the sequences of (a) to (h) linked to each other (either directly or via a spacer

moiety) to give the combination of (i). The compound may further comprise any one of the amino acids sequences, or combinations as described above (in (a) to (i) above), together with additional amino acids or amino acid sequences. The additional amino acids may be sequences from other regions of the Tie-2, c-kit, ILK, VEGF and GRK kinases, sequences that are present in the kinases in the vicinity of the above regions, N-terminal or C-terminal to the sequences of (a) to (d), or sequences which are not present in the Tie-2, c-kit, ILK, VEGF and GRK kinases but were included in the compound in order to improve various physiological properties such as penetration into cells (sequences that enhance penetration through membranes or barriers); decreased degradation or clearance; decreased repulsion by various cellular pumps, improved immunogenic activities, improvement in various modes of administration (such as attachment of various sequences which allow penetration through various barriers, blood brain barrier through the gut, etc.); increased specificity, increased affinity, decreased toxicity, and the like. A specific example is the addition of the amino acid Gly or several Gly residues linked in tandem to N-terminal of the sequence.

The compound may also comprise non-amino acid moieties, such as for example, and preferably, hydrophobic moieties (various linear, branched cyclic, polycyclic or heterocyclic hydrocarbons and hydrocarbon derivatives) attached to the peptides of (a) to (i) to improve penetration through the cells' membranes, various protecting groups, especially where the compound is linear, attached to the compound's terminals to decreased degradation, chemical groups present in the compound to improve penetration or decrease toxic side effects, or various spacers, placed for example, between one or more of the above amino acid sequences, so as to spatially position them in a suitable orientation with respect to each other. The compound of the invention may be linear or cyclic, and cyclization may take place by any means known in the art. Where the compound is composed predominantly of amino acids/amino acid sequences, cyclization may N- to C-terminal, N-terminal to side chain and N-terminal to backbone, C-terminal to side chain, C-terminal to backbone, side chain to backbone and side chain to side chain, as well as

backbone to backbone cyclization. Cyclization of the compound may also take place through the non-amino acid organic moieties.

The association between the amino acid sequence component of the compound and other (non-amino acid) components of the compound may be by covalent linking, by non-covalent complexion, for example, by complexion to a hydrophobic polymer, which can be degraded or cleaved, thus producing a compound capable of sustained release; by entrapping the amino acid part of the compound in liposomes or micelles to produce the final compound of the invention. The association may be by the entrapment of the amino acid sequence within the other component (liposomes, micelles) or the impregnation of the amino acid sequence within a polymer to give the final compound of the invention.

Preferably the compounds comprise an amino acid sequence of (a) to (i) above in association with (in the meaning described above) a moiety for transport across cellular membranes.

The term "*moiety for transport across cellular membranes*" refers to a chemical entity, or a composition of matter (comprising several entities) that causes the transport of members associated (see above) with it through phospholipidic membranes. One example of such moieties are hydrophobic moieties such as linear, branched, cyclic, polycyclic or heterocyclic substituted or non-substituted hydrocarbons. Another example of such a moiety are short peptides that cause transport of compounds attached to them into the cell by, gradient derived, active, or facilitated transport. Other examples of other non-peptidic moieties known to be transported through membranes, such as glycosylated steroid derivatives, are well known in the art. Yet another example are moieties that are endocytosed by membranal receptors such as ligands of the EGF and transferrin receptors. The moiety for the transport across membranes may be a polymer, liposome or micelle containing, entrapping or incorporating the amino acid sequence therein. In the above examples the compound of the invention is the polymer, liposome micelle etc., impregnated with the amino acid sequence.

The term "*a sequence which is a continuous stretch of at least 5 amino acids present ...*" refers to any continuous stretch of at least 5 amino acids, which are present in

a longer amino acid sequence described by reference to the specific positions of Tie-2, c-kit, ILK, VEGF and GRK kinases (see below). For example, if in a c-kit kinase, the positions corresponding to amino acid residues X to Y of c-kit alpha D region are amino acid residues 672 to 694 of that specific kinase, the continuous stretch of at least 5 amino acids may be from amino acid at position 672 to 676, from 673 to 667, from 674 to 678, from 675 to 679, etc. The continuous sequence may also be of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 amino acids, obtained from each of these positions.

The term "*Tie-2*" refers to Tie2 kinase accession Q02763 in NCBI.

The term "*c-kit*" refers to p145 -c kit Accession CAA29548 in NCBI.

10 The term "*ILK*" refers to the kinase in accession number P57043 in NCBI

The term "*VEGF*" refers to vascular endothelial growth factor receptor 2 precursor (VEGFR-2) or protein -tyrosine kinase receptor Flk-1) accession P35968 in NCBI.

The term "*GRK*" refers to GRK2 accession number NP_001610 in NCBI

15 The term "*sequence corresponding to positions ... to ... of Tie-2, c-kit, ILK, VEGF and GRK kinases*" refers to a sequence that matches the sequence appearing in the native kinases in specific positions. The positions of the regions of the kinases beginning in a certain position and ending in another position are indicated in the definition (a) to (d) above.

The term "*wherein up to 40% of amino acids of the native sequence have been replaced with a naturally or non-naturally occurring amino acid or with a peptidomimetic organic moiety*" in accordance with the present invention, concerns an amino acid sequence, which shares at least 60% of its amino acid with the native sequence as described in (a), (b), (c) or (d) above, but some of the amino acids were replaced either by other naturally occurring amino acids, (both conservative and non-conservative substitutions), by non-naturally occurring amino acids (both conservative and non-conservative substitutions), or with organic moieties which serve either as true peptidomimetics (i.e., having the same steric and electrochemical properties as the replaced amino acid), or merely serve as spacers in lieu of an amino acid, so as to keep the spatial relations between the amino acid spanning this replaced amino acid. Guidelines for

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the determination of the replacements and substitutions are given in the detailed description part of the specification. Preferably no more than 30%, 25% or 20%, 15% or 10% of the amino acids are replaced.

The term "*wherein up to 40% of the amino acids have their side chains chemically modified*" refers to a variant which has the same type of amino acid residue as in the native sequence, but to its side chain a functional group has been added. For example, the side chain may be phosphorylated, glycosylated, fatty acylated, acylated, iodinated or carboxyacylated. Other examples of chemical substitutions are known in the art and some given below in the "*Detailed Description*" part of the specification. Preferably no more than 35%, 30%, 25%, 20%, 15% of the amino acids are chemically modified

The term "*up to 20% of the amino have been deleted*" refers to an amino acid sequence which maintains at least 80% of its amino acids residues. Preferably no more than 10% of the amino acids are deleted and more preferably none of the amino acids are deleted.

The term "*provided that at least 50% of the amino acids in the parent protein are maintained unaltered in the variants*" the up to 40% substitution, up to 40% chemical modification and up to 20% deletions are combinatorial, i.e., the same variant may have substitutions, chemical modifications and deletions so long as at least 50% of the native amino acids are identical to those of the native Tie-2, c-kit, ILK, VEGF and GRK kinases sequences both as regards the amino acid residue and its position. In addition, the properties of the parent sequence, in "*modulating angiogenesis*", have to be present in the variant. Preferably at least 60%, 65%, 70%, 75%, 80%, 85% of the amino acids in the parent sequence are maintained in the variant. It should be noted that at times while the parent (native) sequence may modulated angiogenesis in one direction (increase or decrease) the variant may modulate the angiogenesis in another direction (decrease or increase respectively).

When calculating 40% (or 35, 30, 25, 20%) replacements of 20% (or 10%) deletion from sequences, the number of actual amino acids should be rounded

mathematically, so that 40% of an 11-mer sequence (4.4) is four amino acids and 40% of a 12-mer sequence (4.8) is five amino acids.

Typically "*essential amino acids*" are maintained, chemically modified or replaced by conservative substitutions, while non-essential amino acids may be maintained, 5 chemically modified, deleted or replaced by conservative or non-conservative replacements. Generally, essential amino acids are identified by various Structure-Activity-Relationship (SAR) techniques (for example amino acids that when replaced by Ala cause loss of activity are considered "*essential*"), or by using various 3D analysis techniques are replaced by conservative substitution while non-essential amino acids can 10 be deleted or replaced by any type of substitution. Guidelines for the determination of the deletions, replacements and substitutions are given in the "*Detailed Description*" part of the specification.

The term "*region*" refers to a sequence in a specific location is the kinases as specified bellow:

- 15 (a) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 1030 to 1052, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 861 to 885; or a continuous stretch of at least five amino acids present in a native ILK in positions of 382 to 407; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 1097 to 1121; 20 continuous stretch of at least five amino acids present in a native GRK in positions of 383 to 406 (all denoted HJ-loop);
- (b) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 904 to 923, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 25 672 to 694; or a continuous stretch of at least five amino acids present in a native ILK in positions of 271 to 290; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 918 to 939;

continuous stretch of at least five amino acids present in a native GRK in positions of 271 to 291(all denoted α D region);

(c) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 887 to 903, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 655 to 671; or a continuous stretch of at least five amino acids present in a native ILK in positions of 256 to 269; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 900 to 917; continuous stretch of at least five amino acids present in a native GRK in positions of 257 to 271(all denoted B4-B5 region);

(d) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 874 to 891, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 640 to 668; or a continuous stretch of at least five amino acids present in a native ILK in positions of 235 to 258; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 884 to 903 ; continuous stretch of at least five amino acids present in a native GRK in positions of 239 to 260 (all denoted A-region).

The term "*corresponding D-amino acid*" refers to the replacement of the naturally occurring L-configuration of the natural amino acid residue by the D-configuration of the same residue.

The term "*at least one peptidic backbone has been altered to a non-naturally occurring peptidic backbone*" means that the bond between the N- of one amino acid residue to the C- of the next has been altered to non-naturally occurring bonds for example by reduction (to $-\text{CH}_2\text{-NH}-$), alkylation (methylation) on the nitrogen atom, or the bonds have been replaced by non-naturally occurring bonds such as amidic bond, urea bonds, or sulfonamide bond, etheric bond ($-\text{CH}_2\text{-O}-$), thioetheric bond ($-\text{CH}_2\text{-S}-$), or to $-\text{CS-NH}-$. The side chain of the residue may be shifted to the backbone nitrogen to obtain N-alkylated-Gly (a peptidoid).

The term "*in reverse order*" refers to the fact that the sequence of (a) to (f) may have the order of the amino acids as it appears in the native Tie-2, c-kit, ILK, and GRK kinases from N- to the C- direction, or may have the reversed order (as read in the C-to N- direction) for example, if a subsequence of the α D of c-kit DLLNFLRRKRDSF a sequence in a reverse order is FSDRKRRRLFNLLD. It has been found that many times sequences having such a reverse order can have the same properties, in small peptides, as the "*correct*" order, probably due to the fact that the side chains, and not the peptidic backbones are those responsible for interaction with other cellular components. Particularly preferred, are what is termed "*retro inverso*" peptides – i.e., peptides that have both a reverse order as explained above, and in addition each and every single one of the amino acids, has been replaced by the non-naturally occurring D- amino acid counterpart, so that the net end result, as regards the positioning of the side chains, (the combination of reverse order and the change from L to D) is zero change. Such *retro-inverso* peptides, while having similar binding properties to the native peptide, were found to be resistant to degradation.

The term "*therapeutically effective amount*" is the quantity of compound which results in an improved clinical outcome as a result of the treatment as compared with a typical clinical outcome in the absence of the treatment. An "*improved clinical outcome*" results in an individual with the disease experiencing fewer symptoms or complications of the disease, including a longer life expectancy, as a result of the treatment as well as the prevention of the disease before it occurs. The present invention also concerns methods for obtaining the above compounds which compounds can be used in a method for modulating (increasing or decreasing) angiogenesis. Thus the present invention concerns a method for obtaining a compound for the modulation of angiogenesis the method comprising:

- (i) providing a plurality of candidate compounds comprising any one of the sequences of (a) to (i) as defined above;
- (ii) assaying the candidate compounds obtained in (i) in a test assay and determining the level of change in the angiogenesis;

- (iii) selecting those compounds that were able to modulate angiogenesis as compared to the modulation in the same test assay in the absence of the candidate compounds, thereby obtaining compounds capable of modulating angiogenesis.

5 Assays for determining angiogenesis modulating properties will be specified in more detail hereinafter below and in general include modulation of proliferation of endothelial and/or smooth muscle cells; modulation of sprouting of new blood vessels from whole mounts of aortic rings; determination of infiltration of new blood vessels into sponges implanted under the skin (sponge assay); determination of formation of new blood
10 vessels as a result of the implantation of a chip in the cornea. (eye assay) , changes in the formation of new blood vessels which were dissected in the limbs of large animals.

It should be appreciated that some of the compounds comprising the sequences of (a) to (i) above, have better angiogenesis modulating properties than others, and the selection of the compounds which have these properties should be done according to the
15 method as indicated above.

Preferably, the determination of the sequence to be included in the compound and having angiogenesis modulating properties should be carried out with the following steps:

- (i) determining in Tie-2, c-kit, ILK, VEGF and GRK kinases four regions as described in (a) to (d) above
- 20 (ii) determining a continuous stretch of at least 5 amino acids of any of the four regions of Tie-2, c-kit, ILK, VEGF and GRK kinases (i) above, that is shorter than the length of the entire region, and can modulate angiogenesis by: synthesizing a plurality of subsequences (optionally partially overlapping subsequences) having a length of 5-10 amino acids from any of
25 the above four regions; testing those sequences in a test assay for determining modulation angiogenesis , and selecting those sequences that have angiogenesis modulating activities ;
- (iii) determining in the sequences of (i) or in the sequences selected in (ii) above, essential and non-essential amino acids by: preparing a plurality of

modified sequences wherein in each modified sequence a single and different position in the native sequence has been replaced with a test amino acid (preferably with Ala residue); testing those modified sequences in a test assay to determine modulation angiogenesis; those amino acids which when replaced caused a significant change in the modulation of angiogenesis being "*essential amino acids*", and those amino acids which when replaced, did not cause a significant change in the modulation of angiogenesis being "*non-essential amino acids*";

(iv) preparing a plurality of compounds comprising sequences selected from:

- (1) the sequences of (i);
- (2) the sequences selected in (ii);
- (3) the sequences of (i) or the selected sequence of (ii), wherein at least one of the essential amino acids has been chemically modified, replaced by a conservatively substituted naturally or non-naturally occurring amino acid, or by a conservative peptidomimetic organic moiety; and/or at least one of the non-essential amino acids has been chemically modified, deleted, or substituted (conservatively or non-conservatively) by naturally or non-naturally occurring amino acids or a peptidomimetic;
- (4) the sequences of (1) to (3) in a reverse order;
- (5) the sequence of (4) wherein all the amino acids have been replaced by their D-counterpart residues;

said compounds of (iv) being candidate compounds for modulating angiogenesis.

The selection of those compounds which are active from among the candidate compounds can be carried out in a suitable test assay as explained above.

The sequence of the Tie-2, c-kit, ILK, VEGF and GRK kinases can be obtained from amino acid sequence databases in accordance with the NCBI entries given above,, respectively, by the indications of the positions given in (a) to (d) above, and it is possible to locate the above four regions. Although the sequences of the region are not very long

(about 8-40 amino acids long), it is of course desirable to find a shorter (preferably the shortest) subsequence of at least 5 continuous amino acids present within this full region, and use this shorter sequence in the compound of the invention. Finding this short subsequence is a routine procedure, which can be achieved by several possible manners, such as by synthesizing sequences of 5-10 amino acids long, having partially overlapping, or adjacent sequences, and optionally optimizing the chosen sequence (if rather longer sequences are used such as, for example, sequences having a length of 8-10) by sequentially-deleting from one or both of its terminals amino acids until the optimal shorter sequence (not necessarily the shortest but a combination of length and activity should be considered) still capable of modulating angiogenesis, is obtained.

After obtaining shorter subsequences which still have angiogenesis modulating properties, it is necessary to find which amino acids, either in the sequence of the full region but preferably in the sequence of the shorter subsequence, are essential (crucial for the angiogenesis modulating activity) and which are non-essential. This can be done by routine Structure-Activity-Relationship (SAR) analysis, wherein a plurality of sequences are prepared, wherein in each sequence a single (and different) amino acid has been replaced, as compared to the native sequence by a "*test amino acid*" – usually the amino acid residue Alanine (a procedure known as: "*Ala-scan*"). Each of the plurality of sequences is again tested for its properties in modulating angiogenesis. Amino acids which when replaced cause loss, or substantial decrease in the modulating properties (both in the pro-angiogenesis as well as in the anti-angiogenesis aspects) as compared to the native sequence, are considered as "*essential amino acids*". Amino acids which when replaced do not cause a decrease modulating properties of the sequence (as compared to the native sequence) are referred to as "*non-essential*" amino acids (the loss or decrease should be determined by statistically significant manners).

Finally, as a last step, a plurality of sequences is prepared which may comprise either the full native sequence of any of the regions; short subsequence of at least 5 amino acids as appearing in any of the regions; variants of the native sequences wherein at least one essential amino acid has been replaced by conservative substitution by a naturally,

non-naturally occurring amino acid or by a peptidomimetic organic moiety or having been chemically modified; or variants of the native sequence wherein at least one amino acid (present in a non-essential position) has been deleted or chemically modified, or an amino acid in a non-essential position has been replaced by conservative or non-conservative substitution by a naturally occurring, non-naturally occurring, or organic peptidomimetic moiety.

For example, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids may be replaced in the sequence used in the compound of the invention as compared with the native sequences present in the Tie-2, c-kit, ILK, VEGF and GRK kinases. The total combination of replacements, deletions, etc. should be such that the resulting variant shares at least 50% of the amino acids with the native sequence (both as regards the nature of the residue and its position in the sequence).

A notable exception to the above is the use of *retro-inverso* amino acids (in reverse order as compared to the native sequence), where when the peptide is in the reversed order, all of its amino acids are replaced with their D- counterparts.

When preparing the compound, it is possible to proceed by one of two strategies: by one strategy it is possible to test (for properties in modulating angiogenesis) a full compound – i.e., a compound comprising both a candidate sequence, and for example, non-amino acid moieties such as hydrophobic moieties present in one of its terminals. This strategy is generally used where the test assay is intact cells, in tissue (*ex-vivo*) or an *in-vivo* assays where the penetration through membranes, (solved by addition of a hydrophobic moiety), is crucial.

Alternatively, it is possible to first optimize the sequence alone (preferably by testing it in a cell-free system (for example for testing for Tie-2, c-kit, ILK, VEGF and GRK kinases dependent phosphorylation of substrates) so as to first find the best sequence possible, and then add to the optimal sequence other moieties, such as hydrophobic moieties, etc. to improve other properties of the compound as a whole such as for the purpose of improving the penetration into cells, resistance to degradation, etc.

The present invention also concerns compounds for modulation of angiogenesis obtained by any of the above methods.

The present invention further concerns pharmaceutical compositions comprising the above compounds as active ingredients. The pharmaceutical composition may contain
5 one species of compounds of the invention or a combination of several species of the invention.

The pharmaceutical compositions of the invention should be used for modulation of angiogenesis.

The pharmaceutical compositions in the pro-angiogenesis aspect may be used for
10 the treatment of a disease selected from: coronary artery diseases, peripheral artery diseases, endothelial vascular diseases, arteriosclerosis, various processes of wound and tissue healing such as healing of bone, tendon, endothelial lining (such as in ulcers in the stomach), for improving the success rates of cell transplantation techniques, as well as in reconstructive surgery to help re-establish proper blood circulation to the reconstructed
15 tissue.

In the anti-angiogenesis aspects the pharmaceutical compositions may be used for the treatment of a disease selected from: cancer, aged-related macular degeneration (which are many times aggravated by normal neovascularization), diabetic retinopathy (which are also caused by non normal neovascularization), rheumatoid arthritis,
20 psoriasis, obesity, hemangioma (AIDS related), Kaposi sarcoma arteriosclerosis, and restenosis.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example
25 only, with reference to the accompanying drawings, in which:

Fig. 1 is a table showing active compounds of the invention by the name (peptide), region, sequence and the assay in which they were tested (proliferation of endothelial A19 or HEC or SMC, aortic ring, sponge or eye assay);

Fig. 2. shows proliferation results of either endothelial cells (HEC) or smooth muscle cells (SMC) in the presence of compounds comprising c-kit derived peptides (2A), Tie-2 derived peptides(2B), ILK derived peptides (2C), VEGF derived peptides (2D)

Fig. 3 shows changes in neovascularization in aortic ring assay in the presence of
5 compounds comprising c-kit derived peptides (3A), Tie-2 derived peptides (3B), ILK derived peptides (3C), VEGF derived peptides (3D) and GRK derived peptides (3E)

Fig. 4. shows whole mount staining of aortic rings for SMC's actine in the presence of the compounds of the invention:

Fig. 5. shows in vivo results of inhibition of angiogenesis in the sponge assay in the
10 presence of Tie-2 derived peptides.

Fig. 6 shows in vivo results of the stimulation of angiogenesis in the eye in the presence of an ILK-derived peptide.

DETAILED DESCRIPTION OF THE INVENTION

1. Addition of non-peptidic groups to produce the compound of the invention

15 Where the compound of the invention is linear, it is possible to place in any of its terminal various functional groups. The purpose of such functional groups may be for the increase of the angiogenesis modulating properties. The functional groups may also serve for the purpose of improving physiological properties of the compound not related directly to the angiogenesis modulating properties such as: increase the
20 stability, increase the penetration through membranes, optimize the tissue localization of the compound, improve the efficacy, decrease the clearance, decrease the toxicity, improve the selectivity, improve the resistance to repletion by cellular pumps, improve the penetration of the compound through barriers (blood, brain, gut) and the like. For convenience sake the free N-terminal of one of the sequences contained in the
25 compounds of the invention will be termed as the N-terminal of the compound, and the free C-terminal of the sequence will be considered as the C-terminal of the compound (these terms being used for convenience sake). Either the C-terminus or

the N-terminus of the sequences, or both, can be linked to a carboxylic acid functional groups or an amine functional group, respectively.

Suitable functional groups are described in Green and Wuts, *"Protecting Groups in Organic Synthesis"*, John Wiley and Sons, Chapters 5 and 7, 1991, the teachings of which
5 are incorporated herein by reference. Preferred protecting groups are those that facilitate transport of the compound comprising the group into a cell, for example, by reducing the hydrophilicity and increasing the lipophilicity of the compound.

These moieties (the functional-groups) can be cleaved *in vivo*, either by hydrolysis or enzymatically, inside the cell. (Ditter *et al.*, *J. Pharm. Sci.* **57**:783 (1968); Ditter *et al.*,
10 *J. Pharm. Sci.* **57**:828 (1968); Ditter *et al.*, *J. Pharm. Sci.* **58**:557 (1969); King *et al.*, *Biochemistry* **26**:2294 (1987); Lindberg *et al.*, *Drug Metabolism and Disposition* **17**:311 (1989); and Tunek *et al.*, *Biochem. Pharm.* **37**:3867 (1988), Anderson *et al.*, *Arch. Biochem. Biophys.* **239**:538 (1985) and Singhal *et al.*, *FASEB J.* **1**:220 (1987)). Hydroxyl protecting groups include esters, carbonates and carbamate protecting groups. Amine
15 protecting groups include alkoxy and aryloxy carbonyl groups, as described above for N-terminal protecting groups. Carboxylic acid protecting groups include aliphatic, benzylic and aryl esters, as described above for C-terminal protecting groups. In one embodiment, the carboxylic acid group in the side chain of one or more glutamic acid or aspartic acid residue in a compound of the present invention is protected, preferably with a methyl,
20 ethyl, benzyl or substituted benzyl ester, more preferably as a benzyl ester.

In addition, a modified lysine residue can be added to the C-terminal of the compound to enhance biological activity. Examples of lysine modification include the addition of an aromatic substitute, such as benzoyl benzoic acid, dansyl-lysine various derivatives of benzoic acids (difluoro-, trifluoromethy-, acetamido-, dimethyl-,
25 dimethylamino-, methoxy-) or various derivatives of carboxylic acid (pyrazine-, thiophene-, pyridine-, indole-, naphthalene-, biphenyl,), or an aliphatic group, such as acyl, or a myristic or stearic acid, at the epsilon amino group of the lysine residue.

Examples of N-terminal protecting groups include acyl groups (-CO-R1) and alkoxy carbonyl or aryloxy carbonyl groups (-CO-O-R1), wherein R1 is an aliphatic,

substituted aliphatic, benzyl, substituted benzyl, aromatic or a substituted aromatic group. Specific examples of acyl groups include acetyl, (ethyl)-CO-, n-propyl-CO-, iso-propyl-CO-, n-butyl-CO-, sec-butyl-CO-, t-butyl-CO-, hexyl, lauroyl, palmitoyl, myristoyl, stearyl, oleoyl phenyl-CO-, substituted phenyl-CO-, benzyl-CO- and (substituted benzyl)-CO-. Examples of alkoxy carbonyl and aryloxy carbonyl groups include CH₃-O-CO-, (ethyl)-O-CO-, n-propyl-O-CO-, iso-propyl-O-CO-, n-butyl-O-CO-, sec-butyl-O-CO-, t-butyl-O-CO-, phenyl-O-CO-, substituted phenyl-O-CO- and benzyl-O-CO-, (substituted benzyl)-O-CO-. Adamantan-, naphthalen-, myristoleyl, toluen-, biphenyl, cinnamoyl, nitrobenzoyl, toluoyl, furoyl, benzoyl, cyclohexane, norbornane, Z-caproic. In order to facilitate the N-acylation, one to four glycine residues can be present in the N-terminus of the compound.

The carboxyl group at the C-terminus of the compound can be protected, for example, by an amide (i.e., the hydroxyl group at the C-terminus is replaced with -NH₂, -NHR₂ and -NR₂R₃) or ester (i.e., the hydroxyl group at the C-terminus is replaced with -OR₂). R₂ and R₃ are independently an aliphatic, substituted aliphatic, benzyl, substituted benzyl, aryl or a substituted aryl group. In addition, taken together with the nitrogen atom, R₂ and R₃ can form a C₄ to C₈ heterocyclic ring with from about 0-2 additional heteroatoms such as nitrogen, oxygen or sulfur. Examples of suitable heterocyclic rings include piperidinyl, pyrrolidinyl, morpholino, thiomorpholino or piperazinyl. Examples of C-terminal protecting groups include -NH₂, -NHCH₃, -N(CH₃)₂, -NH(ethyl), -N(ethyl)₂, -N(methyl)(ethyl), -NH(benzyl), -N(C₁-C₄ alkyl)(benzyl), -NH(phenyl), -N(C₁-C₄ alkyl)(phenyl), -OCH₃, -O-(ethyl), -O-(n-propyl), -O-(n-butyl), -O-(iso-propyl), -O-(sec-butyl), -O-(t-butyl), -O-benzyl and -O-phenyl.

Preferably the compound includes in the N-terminal a hydrocarbon having a length of C₄-C₂₀ preferably C₆-C₁₈, most preferably C₁₀-C₁₆. Examples of hydrophobic moieties are: merystyl, stearyl, lauroyl, palmitoyl and acetyl etc.

2. Finding the shortest continuous stretch-subsequence of the region

As indicated, the sequences from which the continuous stretch of amino acids are by the following positions :

- 5 (a) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 1030 to 1052, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 861 to 885; or a continuous stretch of at least five amino acids present in a native ILK in positions of 382 to 407; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 1097 to 1121; 10 continuous stretch of at least five amino acids present in a native GRK in positions of 383 to 406 (all denoted HJ-loop);
- 15 (b) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 904 to 923, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 672 to 694; or a continuous stretch of at least five amino acids present in a native ILK in positions of 271 to 290; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 918 to 939; 20 continuous stretch of at least five amino acids present in a native GRK in positions of 271 to 291(all denoted α D region);
- 25 (c) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 887 to 903, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 655 to 671; or a continuous stretch of at least five amino acids present in a native ILK in positions of 256 to 269; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 900 to 917; continuous stretch of at least five amino acids present in a native GRK in positions of 257 to 271(all denoted B4-B5 region);
- (d) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 874 to 891, or a sequence which is a

continuous stretch of at least five amino acids present in c-kit in positions 640 to 668; or a continuous stretch of at least five amino acids present in a native ILK in positions of 235 to 258; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 884 to 903 ;
5 continuous stretch of at least five amino acids present in a native GRK in positions of 239 to 260 (all denoted A-region).

The continuous stretch of at least five amino acids (preferably the shortest possible sequence) can be found by preparing a series of partially overlapping peptides each of 5-10 amino acids and each obtained by synthesizing a sequence that is one position removed
10 from the previous sequence.

For example, if the HJ region of GRK is in positions 383 406, and it is to be desired to prepare 10 mer peptides, then the following, partially overlapping peptides are prepared, a peptide having the sequence 383-392, 384-394, 397-406. The angiogenesis modulating properties are then determined in a relevant assay as will be explained below
15 in the examples. The best 10-mer peptide is then chosen.

For checking whether the 10 mer peptide can be reduced in length, it is possible to either repeat the above procedure (preparing a series of partially overlapping peptides) using for example 5 mer peptides that span the length of the 10 mer peptide, or to shorten the 10 mer peptide by alternately deleting, from each terminal, an amino acid, and testing
20 the angiogenesis modulating properties of the progressively truncated peptides, until the optimal sequence of at least 5, at least 6, at least 7, at least 8, at least 9 mer peptide is obtained, or until verification that longer sequences are required. As the regions are relatively small, the number of different peptides to be tested is also small. For example, for a region α D of c-kit has a length of 23 amino , there is a need to prepare only 15
25 peptides to find the optimal 8 mer peptide. After the best 8-mer peptide is obtained, it is possible to delete sequentially amino acids from one or both terminals of the 8 per peptide for obtaining the shortest sequence of 5, 6 or 7 mer that is still active. For these steps only 19 sequences have to be tested.

3. Identifying essential and non-essential amino acids in the subsequence chosen

A. Ala-Scan

Once the shorter continuous stretch of at least 5 (at least 6, 7, 8, 9, 10, 11 or 12) amino acids has been identified, as explained above, it is necessary to realize which of the amino acids in the stretch are essential (i.e., crucial for angiogenesis modulating activities) and which are non-essential. Without wishing to be bound by theory, in almost every native protein involved in interaction with other cellular components, some amino acids are involved in the interaction (these being the "essential amino acids") and some amino acids are not involved in the interaction (these being "non-essential amino acids"), for example since they are cryptic. A short peptide which is to mimic a region of the Tie-2, c-kit, ILK, VEGF and GRK kinases behaves in the same way as the region when present in the full kinase: some amino acids actually interact with the substrate (or with the other interacting cellular components) and other amino acids merely serve to spatially position the interacting amino acids, but do not participate in the interaction (binding) with the other cellular components.

Essential amino acids have to be maintained (i.e., be identical to those appearing in the native kinase), chemically modified, or replaced by conservative substitutions (see definition below) to obtain variants of the peptides. Non-essential amino acids can be maintained, deleted, chemically modified, replaced by a spacer or replaced by conservative or non-conservative substitutions.

Identification of essential vs. non-essential amino acids in the peptide can be achieved by preparing several peptides that have a shorter sequence than the full region (see Example 2 above) in which each amino acid is sequentially replaced by the amino acid Ala ("*Ala-Scan*"), or sequentially each amino acid is omitted ("*omission-scan*"). This allows to identify the amino acids which angiogenesis modulating properties are decreased by said replacement/omission (these amino acids being defined as "*essential*") and which are replacements/ omissions do not decrease by modulating properties ("*non-essential*") (Morrison *et al.*, *Chemical Biology* 5:302-307, 2001). Another option for

testing the importance of various peptides is by the use of site-directed mutagenesis. Other Structure-Activity-Relationship techniques may also be used.

B. 3D-analysis

Another strategy for finding essential vs. non-essential amino acids is by
5 determining which amino acids of the regions, in the 3D structure of the full kinase, are exposed and which are cryptic. This can be done using standard software such as SPDB Viewer™, "*color by accessibility*" of Glaxo-Wellcome.

Typically cryptic amino acids are non-essential, and exposed or partially
exposed amino acids are more likely to be essential as they are available for
10 interaction with the other cellular components. In addition, if one wishes to "*guess*" theoretically which "*non-conservative*" substitutions in the cryptic region can be tolerated, a good guideline is to "*check*" on a 3D computer model of the full kinase, whether a peptide, superimposed on the full kinase and bearing those changes, still has the overall structure of the region and more importantly, whether the exposed amino
15 acids in the variant (having those substitutions) still overlap the positions of the exposed amino acids in the full kinase. Those non-conservative substitutions, that when simulated on a computer 3D structure (for example using the Tripose™ software) do not cause drastic alterations of the overall shape of the region (i.e., drastic shifting in the positioning of the exposed amino acids) are likely to be non-
20 conservative replacements that can be tolerated, and those substitutions that do cause drastic changes are replacements that are likely to be undesirable.

Thus prior to experimental testing it is possible to reduce the number of tested candidates by computer simulation. Where the 3D structure of a specific kinase is not available in actual crystallography data, it is possible to obtain a "*virtual*" 3D structure
25 of the kinase based on homology to known crystallographic structures using such progress such as CompSer™ (Tripose, USA).

4. Obtaining Variants

The sequence regions of the compounds of the invention may be the native sequences obtained from the Tie-2, c-kit, ILK, VEGF and GRK kinases (preferably the shortest possible sequence from the regions of the kinases), or alternatively
5 variants of the native sequence obtained by deletion, (of non-essential amino acids) or substitution (only conservative substitutions in essential positions, both conservative and non-conservative of non-essential acids), or by chemical modifications of the side chains.

4.1 Deletions and insertions

10 Deletions can occur, in particular deletions of the “*non-essential amino acids*”. Additions may occur in particular at the N-terminal or the C-terminal of any of the amino acids of the sequence. No more than 20%, preferably 10%, most preferably none of the amino acids should be deleted. Insertions should preferably be N-terminal or C-terminal to the sequence of (a) to (h) or between the several sequences linked to
15 each other in (i). However other insertions or deletions are possible. Again, the feasibility of the deletions in creating a peptide which is a good mimic of the native region (when present in the full kinase) can be evaluated virtually by reverting to the 3D-model as described above, and finding which deletions (when the variant is superimposed on the 3D structure of the full kinase) still maintain the exposed side
20 chains in the same orientation as in the native region (when present in the full kinase). This is achieved by superimposing the peptide virtually on the kinase, in the relevant region, so as to determine whether deletions changed drastically the spatial orientation of the exposed side chains.

4.2 Replacements

25 The variants can be obtained by replacements (termed also in the text “*substitutions*”) of any of the amino acids as present in the native kinases. As may be appreciated there are positions in the sequence that are more tolerant to substitutions than others, and in fact some substitutions may improve the activity of the native sequence.

The determination of the essential vs. non-essential positions may be achieved by using "Ala-Scan," "omission scan" "site directed mutagenesis" (all the above being routine SAR procedures) or 3D theoretical considerations as described in 3 above. Generally speaking the amino acids that were found to be "essential" should either be identical to the amino acids present in the native Tie-2, c-kit, ILK, VEGF and GRK kinases, chemically modified, or alternatively substituted by "conservative substitutions" (see below). The amino acids which were found to be "non-essential" might be identical to those in the native peptide, may be substituted by conservative or non-conservative substitutions, may be chemically modified, may be deleted, or replaced by "spacers".

The term "naturally occurring amino acid" refers to a moiety found within a peptide and is represented by -NH-CHR-CO-, wherein R is the side chain of a naturally occurring amino acid.

The term "non-naturally occurring amino acid" (amino acid analog) is either a peptidomimetic, or is a D or L residue having the following formula: -NH-CHR-CO-, wherein R is an aliphatic group, a substituted aliphatic group, a benzyl group, a substituted benzyl group, an aromatic group or a substituted aromatic group and wherein R does not correspond to the side chain of a naturally-occurring amino acid. This term also refers to the D-amino acid counterpart of naturally occurring amino acids. Amino acid analogs are well known in the art; a large number of these analogs are commercially available. Many times the use of non-naturally occurring amino acids in the peptide has the advantage that the peptide is more resistant to degradation by enzymes which fail to recognize this configuration of amino acids.

The term "conservative substitution" in the context of the present invention refers to the replacement of an amino acid present in the native sequence in the specific kinase with a naturally or non-naturally occurring amino or a peptidomimetics having similar steric properties. Where the side-chain of the native amino acid to be replaced is either polar or hydrophobic, the conservative substitution should be with a naturally occurring amino acid, a non-naturally occurring amino acid or with a peptidomimetic moiety which is also polar or hydrophobic (in addition to having the same steric properties as the side-

chain of the replaced amino acid). However where the native amino acid to be replaced is charged, the conservative substitution, according to the definition of the invention, may also be with a naturally occurring amino acid, a non-naturally occurring amino acid or a peptidomimetic moiety which are charged, or with non-charged (polar, hydrophobic) amino acids that have the same steric properties as the side-chains of the replaced amino acids. The purpose of such a procedure of maintaining the steric properties but decreasing the charge is to decrease the total charge of the compound.

For example in accordance with the invention the following substitutions are considered as conservative: replacement of arginine by citrulline; arginine by glutamine; aspartate by asparagine; glutamate by glutamine.

As the naturally occurring amino acids are grouped according to their properties, conservative substitutions by naturally occurring amino acids can be easily determined bearing in mind the fact that in accordance with the invention replacement of charged amino acids by sterically similar non-charged amino acids are considered as conservative substitutions.

For producing conservative substitutions by non-naturally occurring amino acids it is also possible to use amino acid analogs (synthetic amino acids) well known in the art. A peptidomimetic of the naturally occurring amino acid is well documented in the literature known to the skilled practitioner.

When affecting conservative substitutions the substituting amino acid should have the same or a similar functional group in the side chain as the original amino acid.

The following are some non-limiting examples of groups of naturally occurring amino acids or of amino acid analogs are listed below. Replacement of one member in the group by another member of the group will be considered herein as conservative substitutions:

Group I includes leucine, isoleucine, valine, methionine, phenylalanine, serine, cysteine, threonine and modified amino acids having the following side chains: ethyl, n-butyl, $-\text{CH}_2\text{CH}_2\text{OH}$, $-\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}$, $-\text{CH}_2\text{CHOHCH}_3$ and $-\text{CH}_2\text{SCH}_3$. Preferably Group I includes leucine, isoleucine, valine and methionine.

Group II includes glycine, alanine, valine, serine, cysteine, threonine and a modified amino acid having an ethyl side chain. Preferably Group II includes glycine and alanine.

Group III includes phenylalanine, phenylglycine, tyrosine, tryptophan, cyclohexylmethyl, and modified amino residues having substituted benzyl or phenyl side chains. Preferred substituents include one or more of the following: halogen, methyl, ethyl, nitro, methoxy, ethoxy and -CN. Preferably, Group III includes phenylalanine, tyrosine and tryptophan.

Group IV includes glutamic acid, aspartic acid, a substituted or unsubstituted aliphatic, aromatic or benzylic ester of glutamic or aspartic acid (e.g., methyl, ethyl, n-propyl iso-propyl, cyclohexyl, benzyl or substituted benzyl), glutamine, asparagine, CO-NH-alkylated glutamine or asparagine (e.g., methyl, ethyl, n-propyl and iso-propyl) and modified amino acids having the side chain $-(CH_2)_3-COOH$, an ester thereof (substituted or unsubstituted aliphatic, aromatic or benzylic ester), an amide thereof and a substituted or unsubstituted N-alkylated amide thereof. Preferably, Group IV includes glutamic acid, aspartic acid, glutamine, asparagine, methyl aspartate, ethyl aspartate, benzyl aspartate and methyl glutamate, ethyl glutamate and benzyl glutamate.

Group V includes histidine, lysine, arginine, N-nitroarginine, β -cycloarginine, μ -hydroxyarginine, N-amidinocitruline and 2-amino-4-guanidinobutanoic acid, homologs of lysine, homologs of arginine and ornithine. Preferably, Group V includes histidine, lysine, arginine, and ornithine. A homolog of an amino acid includes from 1 to about 3 additional methylene units in the side chain.

Group VI includes serine, threonine, cysteine and modified amino acids having C1-C5 straight or branched alkyl side chains substituted with -OH or -SH. Preferably, Group VI includes serine, cysteine or threonine.

In this invention any cysteine in the original sequence or subsequence can be replaced by a homocysteine or other sulfhydryl-containing amino acid residue or analog. Such analogs include lysine or β -amino alanine, to which a cysteine residue is attached

through the secondary amine yielding lysine-epsilon amino cysteine or alanine-beta amino cysteine, respectively.

The term "*non-conservative substitutions*" concerns replacement of the amino acids as present in the native ie-2, c-kit, ILK, VEGF and GRK kinases by another naturally or
5 non-naturally occurring amino acid, having different electrochemical and/or steric properties, for example as determined by the fact the replacing amino acid is not in the same group as the replaced amino acid of the native kinase sequence. Those non-conservative substitutions which fall under the scope of the present invention are those which still constitute a compound having properties in modulating angiogenesis. Because
10 D-amino acids have hydrogen at a position identical to the glycine hydrogen side-chain, D-amino acids or their analogs can often be substituted for glycine residues, and are a preferred non-conservative substitution.

A "*non-conservative substitution*" is a substitution in which the substituting amino acid (naturally occurring or modified) has significantly different size, configuration and/or
15 electronic properties compared with the amino acid being substituted. Thus, the side chain of the substituting amino acid can be significantly larger (or smaller) than the side chain of the native amino acid being substituted and/or can have functional groups with significantly different electronic properties than the amino acid being substituted. Examples of non-conservative substitutions of this type include the substitution of
20 phenylalanine or cyclohexylmethyl glycine for alanine, isoleucine for glycine, or -NH-CH₂-[(-CH₂)₅-COOH]-CO- for aspartic acid.

Alternatively, a functional group may be added to the side chain, deleted from the side chain, or exchanged with another functional group. Examples of non-conservative substitutions of this type include adding an amine or hydroxyl, carboxylic acid to the
25 aliphatic side chain of valine, leucine or isoleucine, exchanging the carboxylic acid in the side chain of aspartic acid or glutamic acid with an amine or deleting the amine group in the side chain of lysine or ornithine. In yet another alternative, the side chain of the substituting amino acid can have significantly different steric and electronic properties from the functional group of the amino acid being substituted. Examples of such

modifications include tryptophan for glycine, lysine for aspartic acid and $-(\text{CH}_2)_4\text{COOH}$ for the side chain of serine. These examples are not meant to be limiting.

As indicated above the non-conservative substitutions should be of the "non-essential" amino acids.

5 Preferably, the Tie-2, c-kit, ILK, VEGF and GRK kinases may be substituted by benzylamine groups, by biotinylation. Another substitution is di-iodinization of tyrosine. Gly-residues may be substituted by D-isomers especially D-Lys residues.

"Peptidomimetic organic moiety" can be substituted for amino acid residues in the compounds of this invention both as conservative and as non-conservative substitutions.
10 These peptidomimetic organic moieties either replace amino acid residues of essential and non-essential amino acids or act as spacer groups within the peptides in lieu of deleted amino acids (of non-essential amino acids). The peptidomimetic organic moieties often have steric, electronic or configurational properties similar to the replaced amino acid and such peptidomimetics are used to replace amino acids in the essential positions, and are
15 considered conservative substitutions. However such similarities are not necessarily required. The only restriction on the use of peptidomimetics is that the compounds have angiogenesis modulating properties.

Peptidomimetics are often used to inhibit degradation of the peptides by enzymatic or other degradative processes. The peptidomimetics can be produced by organic
20 synthetic techniques. Examples of suitable peptidomimetics include D amino acids of the corresponding L amino acids, tetrazol (Zabrocki *et al.*, *J. Am. Chem. Soc.* **110**:5875-5880 (1988)); isosteres of amide bonds (Jones *et al.*, *Tetrahedron Lett.* **29**: 3853-3856 (1988));

LL-3-amino-2-propenidone-6-carboxylic acid (LL-Acp) (Kemp *et al.*, *J. Org. Chem.* **50**:5834-5838 (1985)). Similar analogs are shown in Kemp *et al.*, *Tetrahedron Lett.* **29**:5081-5082 (1988) as well as Kemp *et al.*, *Tetrahedron Lett.* **29**:5057-5060 (1988),
25 Kemp *et al.*, *Tetrahedron Lett.* **29**:4935-4938 (1988) and Kemp *et al.*, *J. Org. Chem.* **54**:109-115 (1987). Other suitable peptidomimetics are shown in Nagai and Sato, *Tetrahedron Lett.* **26**:647-650 (1985); Di Maio *et al.*, *J. Chem. Soc. Perkin Trans.*, 1687 (1985); Kahn *et al.*, *Tetrahedron Lett.* **30**:2317 (1989); Olson *et al.*, *J. Am. Chem. Soc.*

112:323-333 (1990); Garvey *et al.*, *J. Org. Chem.* **56**:436 (1990). Further suitable peptidomimetics include hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Miyake *et al.*, *J. Takeda Res. Labs* **43**:53-76 (1989)); 1,2,3,4-tetrahydro-isoquinoline-3-carboxylate (Kazmierski *et al.*, *J. Am. Chem. Soc.* **113**:2275-2283 (1991)); histidine isoquinolone
5 carboxylic acid (HIC) (Zechel *et al.*, *Int. J. Pep. Protein Res.* **43** (1991)); (2S, 3S)-methyl-phenylalanine, (2S, 3R)-methyl-phenylalanine, (2R, 3S)-methyl-phenylalanine and (2R, 3R)-methyl-phenylalanine (Kazmierski and Hruby, *Tetrahedron Lett.* (1991)).

4.3 Chemical Modifications

In the present invention the side amino acid residues appearing in the native
10 sequence may be chemically modified, i.e., changed by addition of functional groups to the side chain. The modification may be in the process of synthesis of the compound, i.e., during elongation of the amino acid chain in the process of sequential addition of amino acids, i.e., a chemically modified amino acid is added instead of a naturally occurring one. However, chemical modification of an amino acid when it is present in the compound or
15 sequence (“*in situ*” modification) is also possible.

The amino acid of any of the sequence regions of the compound can be chemically modified (in the peptide conceptionally viewed as “*chemically modified*”) by carboxymethylation, acylation, phosphorylation, glycosylation or fatty acylation. Ether
20 bonds can be used to join the serine or threonine hydroxyl to the hydroxyl of a sugar. Amide bonds can be used to join the glutamate or aspartate carboxyl groups to an amino group on a sugar (Garg and Jeanloz, *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 43, Academic Press (1985); Kunz, *Ang. Chem. Int. Ed. English* **26**:294-308 (1987)). Acetal and ketal bonds can also be formed between amino acids and
25 carbohydrates. Fatty acid acyl derivatives can be made, for example, by free amino group (e.g., lysine) acylation (Toth *et al.*, *Peptides: Chemistry, Structure and Biology*, Rivier and Marshal, eds., ESCOM Publ., Leiden, 1078-1079 (1990)).

4.4 Cyclization of the compound

The present invention also includes cyclic compounds which are cyclic molecules.

A "*cyclic compound*" refers, in one instance, to a compound of the invention in which a ring is formed by the formation of a peptide bond between the nitrogen atom at the N-terminus and the carbonyl carbon at the C-terminus.

"*Cyclized*" also refers to the formation of a ring by a covalent bond between the
5 nitrogen at the N-terminus of the compound and the side chain of a suitable amino acid in the sequence present therein, preferably the side chain of the C-terminal amino acid. For example, an amide can be formed between the nitrogen atom at the N-terminus and the carbonyl carbon in the side chain of an aspartic acid or a glutamic acid. Alternatively, the compound can be cyclized by forming a covalent bond between the carbonyl at the C-
10 terminus of the compound and the side chain of a suitable amino acid in the sequence contained therein, preferably the side chain of the N-terminal amino acid. For example, an amide can be formed between the carbonyl carbon at the C-terminus and the amino nitrogen atom in the side chain of a lysine or an ornithine. Additionally, the compound can be cyclized by forming an ester between the carbonyl carbon at the C-terminus and the
15 hydroxyl oxygen atom in the side chain of a serine or a threonine.

"*Cyclized*" also refers to forming a ring by a covalent bond between the side chains of two suitable amino acids in the sequence present in the compound, preferably the side chains of the two terminal amino acids. For example, a disulfide can be formed between the sulfur atoms in the side chains of two cysteines. Alternatively, an ester can be formed
20 between the carbonyl carbon in the side chain of, for example, a glutamic acid or an aspartic acid, and the oxygen atom in the side chain of, for example, a serine or a threonine. An amide can be formed between the carbonyl carbon in the side chain of, for example, a glutamic acid or an aspartic acid, and the amino nitrogen in the side chain of, for example, a lysine or an ornithine.

25 In addition, a compound can be cyclized with a linking group between the two termini, between one terminus and the side chain of an amino acid in the compound, or between the side chains to two amino acids in the peptide or peptide derivative. Suitable linking groups are disclosed in Lobl *et al.*, WO 92/00995 and Chiang *et al.*, WO 94/15958, the teachings of which are incorporated into this application by reference.

Methods of cyclizing compounds having peptide sequences are described, for example, in Lobl *et al.*, WO 92/00995, the teachings of which are incorporated herein by reference. Cyclized compounds can be prepared by protecting the side chains of the two amino acids to be used in the ring closure with groups that can be selectively removed
5 while all other side-chain protecting groups remain intact. Selective deprotection is best achieved by using orthogonal side-chain protecting groups such as allyl (OAI) (for the carboxyl group in the side chain of glutamic acid or aspartic acid, for example), allyloxy carbonyl (Aloc) (for the amino nitrogen in the side chain of lysine or ornithine, for example) or acetamidomethyl (Acm) (for the sulfhydryl of cysteine) protecting groups.
10 OAI and Aloc are easily removed by Pd and Acm is easily removed by iodine treatment.

5. Pharmaceutical compositions and therapeutical methods of treatment

The compound of the present invention can be used as an active ingredient (together with a pharmaceutically acceptable carrier) to produce a pharmaceutical composition. The pharmaceutical composition may comprise one or a mixture of two or
15 more of the compounds of the invention in an acceptable carrier. The pharmaceutical compositions may also comprise other therapeutically effective ingredients.

The pharmaceutical compositions should be used for the treatment of a disease wherein a therapeutically beneficial effect may be evident through the modulation (increase or decrease) of angiogenesis.

20 In the pro-angiogenesis aspect the pharmaceutical compositions may be used for the treatment of a disease selected from: coronary artery diseases, peripheral artery diseases, endothelial vascular diseases, arteriosclerosis, various processes of wound and tissue healing such as healing of bone, tendon, endothelial lining (such as in ulcers in the stomach or the skin), for improving the success rates of cell transplantation techniques, as
25 well as in reconstructive surgery to help re-establish proper blood circulation to the reconstructed tissue.

In the anti-angiogenesis aspects the pharmaceutical compositions may be used for the treatment of a disease selected from: cancer, aged-related macular degeneration (which are many times aggravated by normal neovascularization), diabetic retinopathy

(which are also caused by non normal neovascularization), rheumatoid arthritis, psoriasis, obesity, hemangioma (AIDS related), Kaposi sarcoma arteriosclerosis, and restenosis.

The term "*therapeutically beneficial effect*" refers either to the prevention of the
5 disease, to alleviation of at least one undesirable effect of the disease, to lessening of the severity of the disease, to the cure of the disease altogether or to slowing the progression of a disease.

A "*therapeutically effective amount*" is the quantity of the compound that results in
an improved clinical outcome as a result of the treatment compared with a typical clinical
10 outcome in the absence of the treatment. An "*improved clinical outcome*" results in the individual with the disease experiencing fewer symptoms or complications of the disease, including a longer life expectancy, less pain, longer disease free periods, and decrease of any of the manifestations of the disease, improved well being, as a result of the treatment as well as the prevention of the disease before it occurs.

15 The amount of compound administered to the individual will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. Typically, a therapeutically effective amount of the compound can range from about 1 mg per day to
20 about 1000 mg per day for an adult. Preferably, the dosage ranges from about 1 mg per day to about 100 mg per day.

The compounds of the present invention can be administered parenterally. Parenteral administration can include, for example, systemic administration, such as by intramuscular, intravenous, subcutaneous, or intraperitoneal injection. The compounds
25 may also be administered locally. For example anti-angiogenesis compounds used to decrease the formation of new blood vessels into the tumor site, may be injected directly into the tumor. Pro-angiogenesis compounds may be administered directly to the site of coronary blockage to encourage formation of new blood vessels that bypass the blocked region. The pro-angiogenesis compounds may also be administered to the site of the

reconstructive surgery to speed the process of formation of new blood vessels at the site. The compounds of the invention may also be administered topically for example for the treatment of external ulcers in the form of salves, ointments, solutions etc. suitable for topical administration. Compounds which resist proteolysis can be administered orally,
5 for example, in capsules, suspensions or tablets. The compound can also be administered by inhalation or insufflations or via a nasal spray.

The compound can be administered to the individual in conjunction with an acceptable pharmaceutical carrier as part of a pharmaceutical composition for treating the diseases discussed above. Suitable pharmaceutical carriers may contain inert ingredients
10 which do not interact with the compounds. Standard pharmaceutical formulation techniques may be employed such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-
15 buffered saline, Hank's solution, Ringer's-lactate and the like. Methods for encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art (Baker, *et al.*, *Controlled Release of Biological Active Agents*, John Wiley and Sons, 1986). The compound may be also resources for administration to bone, or in the form of salve, solution, ointment, etc. for topical administration.

20 6. Preparation of the Compounds

Peptide sequences for producing any of the sequences of the compounds of the invention may be synthesized by solid phase peptide synthesis (e.g., t-BOC or F-MOC) method, by solution phase synthesis, or by other suitable techniques including combinations of the foregoing methods. The t-BOC and F-MOC methods, which are
25 established and widely used, are described in Merrifield, *J. Am. Chem. Soc.*, **88**:2149 (1963); Meienhofer, *Hormonal Proteins and Peptides*, C.H. Li, Ed., Academic Press, 1983, pp. 48-267; and Barany and Merrifield, in *The Peptides*, E. Gross and J. Meienhofer, Eds., Academic Press, New York, 1980, pp. 3-285. Methods of solid phase peptide synthesis are described in Merrifield, R.B., *Science*, **232**:341 (1986); Carpino, L.A. and Han, G.Y.,

J. Org. Chem., **37**:3404 (1972); and Gauspohl, H. *et al.*, *Synthesis*, **5**:315 (1992)). The teachings of these references are incorporated herein by reference.

As indicated above the compounds of the invention may be prepared utilizing various peptidic cyclizing techniques. Methods of cyclizing compounds having peptide sequences are described, for example, in Lobl *et al.*, WO 92/00995, the teachings of which are incorporated herein by reference. Cyclized compounds can be prepared by protecting the side chains of the two amino acids to be used in the ring closure with groups that can be selectively removed while all other side-chain protecting groups remain intact. Selective deprotection is best achieved by using orthogonal side-chain protecting groups such as allyl (OAI) (for the carboxyl group in the side chain of glutamic acid or aspartic acid, for example), allyloxy carbonyl (Aloc) (for the amino nitrogen in the side chain of lysine or ornithine, for example) or acetamidomethyl (Acm) (for the sulfhydryl of cysteine) protecting groups. OAI and Aloc are easily removed by Pd and Acm is easily removed by iodine treatment.

Other modes of cyclization (beyond N- to C- terminal cyclization) may include: N- to backbone cyclization, C- to backbone cyclization, N- to side chain cyclization, C- to side chain cyclization, backbone to side chain cyclization, backbone to backbone cyclization and side chain to side chain cyclization.

7. Determination of angiogenesis modulating properties

It should be appreciated that some of the compounds that comprise sequences (a) – (i) above have better properties in modulating angiogenesis than others. Some of the conservative substitutions in the essential positions may diminish the angiogenesis modulating properties, while other such conservative substitution in the essential positions may improve these properties. The same is true also for deletions, substitutions (both conservative and non-conservative) in non-essential positions, as well as to chemical modifications of the side chains (in any position) or insertions. In addition the type and size of the non-amino acid portion of the compounds, such as a hydrophobic moiety in one of its terminals, may diminish or increase the angiogenesis modulating properties. The angiogenesis modulating properties may be determined by using one of the assays below.

7.1 Cellular Assay

An indication on the angiogenesis modulating properties may be by the determination of the change of the proliferation of endothelial cells or Smooth Muscle Cells (SMC) (for example using the methylene blue technique). However lack of effect on the proliferation is not conclusive as regards lack of effect on the angiogenesis, as many times angiogenesis is changed even without change in the proliferation of the endothelial cells for example by interruption/inhibition of the endothelial and/or smooth muscle cell-cell interactions and their arrangement in regular structures.

The activity of the candidate compound is assessed and compared with a suitable control, e.g., the proliferation of the same cells incubated under the same conditions in the absence of the candidate compound (or in the presence of a control compound). A greater or lesser proliferation as compared with the control indicates that the candidate compound modulates endothelial and/or smooth muscle cell proliferation alluding to a possible effect (that has to be verified) on angiogenesis.

7.2 Phosphorylation of substances

It is possible to assess the kinase activity and the changes in this activity as compared to control, by determining the phosphorylation level of the downstream signal transduction pathway members substrate proteins of Tie-2 (PKB, PKC, Dok-R, Nck) or of the c-kit kinase (AKT, PI3K/PKB, Grb2/Sos/Ras/Raf/MEK/ERK, Lyn, JAK2, STAT1/5), ILK, (PKB p-ser 473), VEGF (PI3K/PKB, p38MAPK, FAK/Paxillin, Src, PLC γ /PKC/Raf/MEK/ERK, eNOS) and GRK (GPCR's). Cells known to express the above kinases are incubated with a candidate compound under condition that modulate the kinase-associated activity. Then the cells are lysed, the protein content of the cells is obtained and separated on a SDS-PAGE. The downstream substrates can be identified by use of suitable molecular weight markers and suitable antibodies. The level of downstream substrates phosphorylation can be determined by using anti-phosphotyrosine antibodies or anti Ser/Thr antibodies, either conjugated to a suitable label or further reacted with a label-bearing antibody (see Fujimoto *et al.*, *Immunity*, **13**:47-57 (2000)).

Alternatively phosphorylation may be determined in a cell-free system by incubating each kinase, its substrate for example as specified above, and candidate compounds, in the presence of ATP under conditions enabling phosphorylation.

The proteins are then subjected to SDS-PAGE, transferred to nitrocellulose
5 followed by immunoblotting by anti-phosphotyrosine antibody. Alternatively it is possible to use [γ - 32 P] ATP and quantify the amount of radioactivity incorporated in the substrate (See Fujimoto *et al.*, *The J. of Immunol.* 7088-7094 (1999)).

7.3. Tissue or *in vivo* Assays

The following are suitable *in vivo* or *ex vivo* modes for angiogenesis:

- 10 1) Aortic ring –where sprouting of newly formed blood vessels is tested whole mounts of aortic rings obtained from rats (see below)
- 2) Eye assay –where formation of new blood vessels in the cornea of the animal as a response to an FGF-implanted pellet is determined.
- 3) Sponge assay – where infiltration of new blood vessels into a sponge
15 implanted s.c. in an experimental animal is determined
- 4) Regression of pre-formed vasculature in tumors (Benjamin L.E. and Keshet E. *PNAS*, 94, 8761-8766, (1997))
- 5) Re-establishment of circulation in dissected vessels in the limbs of experimental animals

20 **EXPERIMENTAL PROCEDURES:**

1. Aortic ring assay:

We used the modification of previously described assay (Nicosia, R.F. and Ottinetti, A. "Growth of microvessels in serum-free matrix culture of rat aorta." *Lab. Invest.* 63:115-122. 1990) for capillary growth *ex vivo*. The assay is based on the
25 phenomenon of endothelial cells proliferation from freshly prepared aorta placed into collagen matrix gel, that finally formed new capillary system.

1.1 Collagen extraction.

Collagen solution was prepared by extraction from rat tendons (modification of the described assays - Strom, S.C. and Michalopoulos, G. "Collagen as a substrate for cell growth and differentiation." Methods Enzymol. 82:544-55, 1982). For this purpose, rat tails were taken from SABRA rats that were sacrificed in the lab during the different experiments. The tails are stored frozen at -20°C until the day of extraction. About 15 tails are used to produce 1 liter of collagen.

Rat tails were thawed and a section of skin from proximal end of each tail was removed using bone clippers. Remaining skin was pulled away while keeping the tail intact to reveal the white tendons. Tails were washed with DDW. We used two pairs of artery forceps to dissect out tail tendons. Starting from the tip of the tail, the tail was clamped at its end with the artery forceps and using the other clamp approximately 2-3 cm away from another pair, the tail was bended until the vertebrae break and tendons come loose from the remaining of the tail. This procedure was repeated several times toward the distal end of the tail. Tendons were cut, washed twice with PBS and placed into Petri dish with 70% Ethanol for sterilization and dehydration. Remaining blood vessels were removed before sterilization. Petri dish with tendons is placed under UV and ethanol is evaporated during overnight incubation.

Then collagen fibers were weighted and solubilized in sterile 0.1% solution of Acetic Acid in DDW* (1-2 gr per 0.5 liter). Solution was stirred slowly at 4°C during 48h. Undissolved tendons are then discarded by centrifugation (16,000-20,000g, 30'). Collagen Stock solution is stored at -20°C . Prior to storage, the collagen was assayed using the polymerization reaction described below. The polymer was examined for both viscosity and cleanliness from remaining tendon particles. Too viscous collagen was diluted in acetic acid. Too "dirty" is being centrifuged again, at higher speed.

The collagen matrix gel was obtained by simultaneously raising of pH and ionic strength of collagen solution (Elsdale and Bard, 1972). Working solution for this purpose was prepared by the following proportion:

Collagen Stock solution – 7parts,

MEMx10 (Minimum Essential Medium Eagle concentrated x10 (obtained from Beit Haemec, cat.01-025-5A)) – 1part,
Na Bicarbonate 0.15M solution – 2parts.

1.2 Experimental animals.

5 The experiments were carried out on two lines of animals – Sabra and Sprague-Dawley rats, males of 200-250 gr-body weight (1-2 month old). Rats were acclimated to vivarium for 4-10 days before their use in study protocol. Each group (from 2 to 4) of the animals was housed in separate cage and fed food and water ad libitum.

1.3 Preparation of aortic rings.

10 Animals were sacrificed by decapitation after ether anesthesia. Thoracic aortas were dissected and immediately transferred to a Petri dish with bio-mpm-1. The fibroadipose tissue and small vessels around aorta was carefully removed under a dissecting microscope, 1 mm-long aortic rings were sectioned and extensively (2-3 times) rinsed in bio-mpm-1 containing antibiotics (% cat.) (Nicosia and Ottinetti, 1990).

1.4 Embedding of aortic rings.

15 The collagen matrix gel solution (see above) was added to 24-well plate (0.4 ml to each well) and collagen polymerization was allowed for 15 min at 37°C. The aorta rings were transferred to the collagen gel solution (in order to glue them to the bottom layer of the gel) and then placed to the center of each well. In mice, 3 rings are embedded in the same well. After 15 min incubation of the plates at 37°C another 0.4 ml of the collagen solution was carefully added on the top of the ring. After the gel is formed 0.4 ml of Serum-Free Endothelial Growth Medium (Rhenium, cat. 17601-030) or bio-mpm-1 (Beit Haemeq cat# 05-060-1) was added to each well. Rings were incubated in 37°C and 10% CO₂. Medium is replaced every 2-3 days.

1.5 Addition of Compounds

25 Compound of the invention were added next day after collagen gel formation in concentration 10-20 uM. compounds were dissolved for 10 mM stock in 100% DMSO.

Dilution in 0.1% BSA in PBS to 400 uM stock were done for each AR experiment de novo. The compounds were re-added to AR during each medium changing till well-developed capillary system in control or experimental wells.

1.6 Experimental treatment.

5 **Fixation:** Fixation was carried out by adding of Buffered Formalin 4% (was obtained from BioLab, cat.66554) 1.5ml in each well. An hour later solution was changed to fresh Formalin in order to better fixation (overnight).

Staining: We used Crystal Violet (Gencian Violet) 0.02% in Ethanol Abs. as staining solution (1ml for each well overnight at room temperature). After staining, wells
10 were washed massively with 0.02% Sodium Azide water solution during two or three days till transparent matrix.

1.7 Analysis of results

 Digital pictures were analyzed using ImageProTM software. The total capillary length of at least 6 AR was measured. Dividing the total capillary length to AD diameter
15 normalized the data.

2. Eye assay.

 We used a modification of the assay previously described in "A model of angiogenesis in the mouse cornea." B.M. Kenyon, E.E. Voest, C.C. Chen, E. Flynn, J. Folkman and R.J. D'Amato Investigative Ophthalmology & Visual Science, July 1996, Vol.
20 37, No.8 1625-1632 . The assay is based on the phenomena of formation of new blood vessels in the cornea of mice in response to the implantation in the cornea of a pellet impregnated with FGF.

2.1 FGF pellets preparation.

1. Weight Out: - 120 mg hydron into sterile screwcup
25 - 10 mg sterile sucralfate into Eppendorf.
2. Add 1 ml 100% ethyl alcohol to hydron. Vortex till clear (few minutes).

3. Add 20 ng/20 μ l bFGF to sucralfate- vortex, spidvac for 5 min (more if needed).
4. Add - 10 μ l hydron solution to sucralfate pellet.
5. Mix with sterile spatula.
- 5 6. Using spatula, remove mixture from tube and smear into nylon square (very quick).
7. Coat each side with hydron - light coat.
8. Stand it on dish to dry - glue the mesh with tape to the bottom and to the wall of the plate - pellets must stay in the air (using bacteriological 100 mm
10 plates - less charged).
9. Dry till firm - 30 min
10. - Store at -20°C till implantation procedure.
11. Pull trends releasing pellets.
12. Choose the pellets with the same size- the white pellets are the FGF
15 containing, the transparent ones are hydron only.

2.2 Materials:

1. bFGF - human recombinant.
2. Sucralfate - sucrose octasulfate - aluminium complex Sigma cat# S-0652
3. Hydron - soluble poly-2-hydroxyethylmethacrylate cat# - 97001
- 20 4. Mesh - Sefar America Kansas city, MO 64119 - 3120 Cat# 03-300/51,40 inches wide

Solution for FGF and peptides (solution S):

- 20 mM sodium citrate
- 1mM EDTA
- 25 - 9% sucrose
- pH 5.0

2.3 Peptide and peptide + FGF pellets preparation.

1. To prepare:

- Hydron concentrated stock solution (120 mg/ml in ethanol Abs.)

- Hydron diluted stock solution (60 mg/ml in ethanol Abs.)

5 - sucralfate stock solution (500 mg/ml in solution S sterile !).

- 10 mg peptide.

2. Add 30 µl of solution S to peptide (or 10 µl of solution S and 20 µl of FGF).

3. Add 20 µl of sucralfate stock solution - vortex, speedvac for 5 min (more if needed).

10 4. Add - 20 µl hydron diluted stock solution to sucralfate + FGF +peptide pellet.

5. Mix with sterile spatula.

6. Using spatula, remove mixture from tube and smear into nylon square (very quick).

15 7. Coat each side with hydron concentrated solution - light coat.

8. Stand it on dish to dry - glue the mesh with tape to the bottom and to the wall of the plate - pellets must stay in the air (using bacteriological 100 mm plates - less charged).

9. Dry till firm - 30 min.

20 10. Store at -20°C till implantation procedure.

11. Pull trends releasing pellets.

12. Choose the pellets with the same size- the white pellets are the FGF containing, the transparent ones are hydron only.

2.4 Implantation procedure.

25 Mice were anesthetized with Ketamin/Rompun Solution (for dosage and solutions preparation see standard anesthetic procedure). The eye is topically anesthetized with Localin (Benoxynate HCl 0.4%, Dr.Fisher, Pharmaceutical Labs. P.O.B. 39071, Tel-Aviv 61390). Using a binocular microscope, a central intrastromal linear keratotomy is performed with a surgical blade parallel to the insertion of the lateral rectus muscle.

Approximate length of the cut is 0.5-0.6 mm length. The globe is proptosed with a forceps. A lamellar micropocket is dissected toward the temporal limbus using the modified von Graefe knife 2×30 mm. All following procedures must be done without proptosis. A pellet is placed on the corneal surface in the base of the pocket and advanced
5 to the temporal end of the pocket with one arm of the forceps. The distance between pellet and limbal vessel is measured. For FGF containing pellets, the pocket must be extended within 1 mm of the temporal limbus. Antibiotic and antimycotic ointment are applied to the operated eye to prevent infection and additional irritation of the cornea.

2.5 Experimental procedure

10 Measurements were done at 5th and 7th days after implantation. In the case of high stimulation inspections were done twice a week till full degradation. The maximum vessel length of neovascularization zone, extending from the limbal vessel toward the pellet was measured. The econtiguous circumferential zone of neovascularization was measured as clock hours (where 30° are equals 1 clock hour.)

15 3. Air sac model ('sponge assay').

We used the modification of previously described assays (J.Lichtenberg, C.A.Hancen et al. "The rat subcutaneous Air Sac Model: a new and simple method for in vivo screening of angiogenesis." Pharmacology and Toxicology 1997. 81, 280-284, A.P. Lage and S.P. Andrade "Assessment of angiogenesis and tumor growth in
20 conscious mice by a fluorometric method.").

3.1 Experimental animals.

The experiments were done at BalbC mice, males of 6-7 week age. Mice were acclimated to vivarium for four days before their use in study protocol. Each group (from 8 to 10) of the animals was housed in separate cage and fed food and water ad
25 libitum. The animals were observed daily for clinical signs and body weights were recorded twice weekly.

3.2 Implant.

Sponge pieces was used for subcutaneous implantation. The sponges were sterilized by autoclaving at 121°C for 15 min. The cellulose sponge SpontexR (manufactured by Spontex S.A., Beauvais, France) has previously been used in an in vivo experiments for the study of cartilage degradation (Bishop et al. "A novel model of cartilage segregation." J.Pharmacol.Toxicol.Meth. 1993, 30, 19-25.).

3.3 Air sac formation.

4-5 ml of air were introduced dorsally by subcutaneous injection using a 27 gauge needle to produce an air sac located in the middle of the back. The air sacs were re-inflated every forth day. The wall of the air sac became progressively thicker with time, and after approximately 10 days a sufficient lining of cells had been established. The implantation of the sponge was made not sooner than 10 days after induction of air sac.

3.4 Implantation technique.

All mice received intraperitoneal injection of anesthetic solution 0.1 ml per animal before implantation. Solution was prepared at the day of the treatment as followed: 0.85 ml of Ketamin 100 mg/ml (was obtained from Fort Dodge, NDS 0856-2013-01), 0.15 ml of Rampun 2% (Xylazine HCl 10% , obtained from "Bar-Ilan", B-8049), 1 ml PBS. After injection animals was shaved, marked and separate to the groups.

Each animal was placed on its abdomen and the skin of the back was washed with Ethanol 70%. A 0.5-0.7 cm incision was made through the skin covering the air sac. Blunt dissection was used to open a 1 cm deep cavity towards the cranial base of the air sac by careful separation of the skin from the membrane. A sponge implant (0.5 x 0.5 x 0.2 cm) was carefully inserted to the pocket on the membrane away from the incision. The incision was closed by 2-3 sutures using DexonR 3-0.

3.5 Angiogenic response.

The animals were sacrificed by ether aspiration. The overlying skin was carefully removed to expose the transparent membrane and not to rupture the air sac. The extent vascular proliferation was scored *in situ*. The subjective scoring was taken
5 by following criteria:

- 0 transparent membrane without vascularization (blank membranes without implant).
- 1+ slight background vascularization.
- 2+ few vessels reach the sponge.
- 10 3+ many vessels reach the sponge with beginning of penetration in the implant.
- 4+ very intensive formation of the new vessels with reach and penetration of implant.

After *in situ* scoring the implant from each animal was cut into two equal
15 portions. The first portion was placed in the plastic vial containing 4% Buffered Formalin (for histological sections and further microscopical examination), the second one was measured for hemoglobin concentration

4. Proliferation assay.

Endothelial cells (HEC) or Smooth muscle cells (SMC) were obtained from ATCC.
20 These cell lines were grown in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), glutamine (2 mM) and 10% endotoxin free bovine cell serum (Hyclone).

A suspension of the cells at 2×10^4 cells/ml was prepared in the above-described culture mediums and distributed 0.180 ml per well (about 4000 cells/well) in the wells of
25 96 well, flat bottom, tissue culture microtiter plates.

A series of compounds stock solutions were prepared by diluting a 10 mM solution of the compound in 100% DMSO with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) to a concentration of 400 µM. These solutions were labeled DMSO. In many instances, 40 µl of the 10 compound in DMSO solution was mixed with

160 μ l of 2M NH_4HCO_3 and heated for 40 minutes at 100°C. The resultant solution was then diluted to 400 μ M in PBS containing 0.1% BSA. These compounds stock solutions were labeled "tbi". The concentration of compound in each stock solution was adjusted to nine times the desired concentration of the compound in the assay mixture. 0.020 ml of each compound stock solution was added to the corresponding wells about 2 hours after cell addition, with six replicates for each concentration. In addition, PBS containing 0.1% BSA solution with no added compound was used as a control. The plates were incubated for 72-80 hours at 37°C in a 10% CO_2 humidified incubator. This formulation was termed "tbi", and served as a vehicle and as control.

The plates were labeled and the medium discarded. The wells were fixed with 4% formaldehyde PBS (PBS buffered with 10% formalin from Fisher Scientific; Catalog No. HC200-1) (0.2 ml/well) for at least 30 minutes. The wells were washed one time with borate buffer (0.2 ml/well) (0.1M, pH 8.5). Freshly filtered 1% methylene blue solution (0.60 ml/well) was then added to the wells and incubated for 10 minutes at room temperature. The wells were then washed five times with tap water, after which the wells were dried completely. 0.20 ml/well of 0.1 N HCl was added to extract the color. After overnight extraction, the O.D. was read at 630 nm to determine the number of cells per well. The procedure for counting cells is described in greater detail in Oliver *et al. J. Cell Sci.*, 92: 513 (1989), the teachings of which are incorporated herein by reference.

Whole mount staining of the aortic rings.

For whole mount Smooth Muscle Actin (SMA) FITC staining:

1. Fixation with 1% paraformaldehyde for 30 min.
2. Rinsing with PBS overnight.
3. Block with PBS + 1% BSA overnight + tritonX 100 0.01%.
4. Incubate with rat anti-mouse CD31 (BD PharMingen Catalogue 01951D, rat monoclonal anti-pecam, clone MEC13.3.) 1:500 dilution overnight.
5. Wash with PBS X 3 times. Time over 8-10 hrs.

ABCAM mouse monoclonal to alpha actin (SMA) (FITC) Clone 1A4 ab8211-100 lot:8393 ABCAM Ltd, Cambridge.

Example 1: Modulation of smooth muscle cell and endothelial cell proliferation by the compounds of the invention.

5 The assay was performed as described above in "*Experimental Procedures*". SMC, or endothelial cells (HEC) were incubated with varying concentrations of the compounds of the invention for 4 days, and than were fixated and stained with methylene blue. OD was quantified using an Elisa Reader to give an indication of the number of cells as compared to control.

10 The results are shown in Fig. 2A (for compounds comprising c-kit derived peptides), 2B (for compounds comprising Tie-2 derived peptides), and 2C (for compounds comprising ILK derived peptides), 2D (for compound containing VEGF derived peptides) and. As can be seen c-kit and Tie-2, and VEGF derived peptides inhibited SMC or SMC endothelial cell proliferation, respectively, in a dose dependent
15 manner while compounds comprising ILK derived peptide stimulated cellular proliferation in a dose dependent manner.

Example 2: Modulation of angiogenesis in Aortic ring assay by the compounds of the invention

20 Aortic ring assay was performed as described above in "*Experimental Procedures*" with compounds comprising the peptides of the invention. The results of all compounds tested are shown in a table of Fig. 3.

25 Fig. 3 shows pictures of aortic rings incubated with and without 10 uM of a compound comprising c-kit derived peptide (Fig. 3A), with varying concentrations of compounds comprising Tie-2 derived peptides (Fig. 3B), with and without 10 μ M of a compound comprising GRK derived peptide. (Fig. 4E), and with and without 10 μ M of a compound comprising ILK derived peptide (Fig. 3C) and with and without VEGF derived peptide (Fig. 3D).

As can be seen compounds comprising c-kit, GRK, and Tie-2 and VEGF derived peptides inhibited angiogenesis of aortic rings, while compounds comprising ILK derived peptides stimulated angiogenesis.

Example 3:

5 Example 4: *In vivo* model modulation of angiogenesis in a sponge assay, by the compounds of the invention

Air-sac assay was performed as described in "*Experimental Procedures*". Sponges soaked with 20-50 ul of peptide stock solution (400 uM 0.1% BSA 4% DMSO in PBS) were implanted s.c for two weeks, and than removed and stained for
10 hemoglobin by H&E staining. The results are shown in Fig. 5 for sponges containing compound with Tie-2 derived peptides.

As can be seen the sponge containing the Tie-2 derived peptides features a significantly lower number of penetrated blood vessels as compared to control, indicating that a compound comprising a Tie-2 derived peptides was able to inhibit
15 angiogenesis in an *in vivo* model.

Example 5: *In vivo* modulation of angiogenesis in the eye by the compounds of the invention.

The eye assay was performed as detailed in "*Experimental Procedures*", wherein in one eye of the mice a pellet with FGF only was implanted while in the
20 other eye a pellet with a compound comprising an ILK derived peptide was used.

The results are shown in Fig. 6 wherein in Fig. 6A the picture is of the eye after 5 days of implantation of the FGF-containing pellet, Fig. 6B is the same eye after 15 days, and Fig. 6C is a picture of the eye implanted with FGF containing chip and administered with an ILK-derived peptide.

25 As can be seen in the FGF-containing implanted pellet after 5 days there was a significant formation of blood vessels (Fig. 6A) that were retracted, and almost disappeared on day 15 (Fig. 6B). Against this the eye administered also with an ILK-derived peptides maintained the vasculature also on day 12 (Fig. 6C). This results

indicating that the compound of the invention is capable of modulating angiogenesis by stabilizing newly formed blood vessels for longer periods of time as compared to control.

CLAIMS:

1. A method for the modulation of angiogenesis comprising contacting blood vessels with an effective amount of a compound comprising a sequence selected from:

- 5 (a) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 1030 to 1052, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 861 to 885; or a continuous stretch of at least five amino acids present in a native ILK in positions of 382 to 407; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 1097 to 1121; continuous stretch of at least five amino acids present in a native GRK in positions of 383 to 406 (all denoted HJ-loop);
- 10 (b) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 904 to 923, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 672 to 694; or a continuous stretch of at least five amino acids present in a native ILK in positions of 271 to 290; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 918 to 939; continuous stretch of at least five amino acids present in a native GRK in positions of 271 to 291(all denoted α D region);
- 15 (c) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 887 to 903, or a sequence which is a continuous stretch of at least five amino acids present in c-kit in positions 655 to 671; or a continuous stretch of at least five amino acids present in a native ILK in positions of 256 to 269; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 900 to 917; continuous stretch of at least five amino acids present in a native GRK in positions of 257 to 271(all denoted B4-B5 region);
- 20 (d) a sequence which is a continuous stretch of at least five amino acids present in a native Tie2, in positions of 874 to 891, or a sequence which is a
- 25

continuous stretch of at least five amino acids present in c-kit in positions 640 to 668; or a continuous stretch of at least five amino acids present in a native ILK in positions of 235 to 258; or a continuous stretch of at least five amino acids present in a native VEGF in positions of 884 to 903 ; continuous stretch of at least five amino acids present in a native GRK in positions of 239 to 260 (all denoted A-region);

(e) a variant of a sequence according to any one (a) to (d) wherein up to 40% of the amino acid of the native sequence have been replaced with a naturally or non-naturally occurring amino acid or with a peptidomimetic organic moiety; and/or up to 40% of the amino acids have their side chains chemically modified; and/or up to 20% of the amino acids have been deleted; provided that at least 50% of the amino acids in the parent sequence of (a) to (d) are maintained unaltered in the variant, and provided that the variant has autoimmune disease improving properties;

(f) a sequence of any one of (a) to (e) wherein at least one of the amino acids is replaced by the corresponding D- amino acid;

(g) a sequence of any one of (a) to (f) wherein at least one of the peptidic backbones has been altered to a non-naturally occurring peptidic backbone;

(h) a sequence being the sequence of any one of (a) to (g) in reverse order; and

(i) a combination of two or more of the sequences of (a) to (h).

2. A method according to Claim 1, wherein the sequence is selected from:

(i) a sequence which is a continuous stretch of at least five amino acids selected from: a sequence present in native c-Kit in position 672-694-of the kinase , a sequence present in Tie2 in positions 904-923 of the kinase, a sequence present in VEGF in positions 918-939 of the kinase (α D region) a sequence present in ILK in positions 382-407 of the kinase; and a sequence present in GRK2 in positions 383-406 of the kinase (HJ-loop);

(ii) a variant of a sequence according to (i) wherein up to 40% of the amino acid of the native sequence have been replaced with a naturally or non-

naturally occurring amino acid or with a peptidomimetic organic moiety; and/or up to 40% of the amino acids have their side chains chemically modified; and/or up to 20% of the amino acids have been deleted; provided that at least 50% of the amino acids in the parent sequence of (i) are maintained unaltered in the variant, and provided that the variant has autoimmune disease improving properties ;

(iii) a sequence of any one of (i) or (ii) wherein at least one of the amino acids is replaced by the corresponding D- amino acid;

(iv) a sequence of any one of (i) to (iii) wherein at least one of the peptidic backbones has been altered to a non-naturally occurring peptidic backbone;

(v) a sequence being the sequence of any one of (i) to (iv) in reverse order; and a combination of two or more of the sequences of (i) to (v).

3. A method according to Claim 2, wherein the position of (I) are positions 926 to 937 of VEGF, 909-920 of Tie-2 or 677-689 of c-kit (sub-sequences of alpha D region).

4. A method according to Claim 1 for the increase of angiogenesis, wherein the sequence is selected from:

(i) a sequence which is a continuous stretch of at least five amino acids present in the native ILK kinase in positions 382-407(HJ-loop);

(ii) a variant of a sequence according to any one of (i) wherein up to 40% of the amino acid of the native sequence have been replaced with a naturally or non-naturally occurring amino acid or with a peptidomimetic organic moiety; and/or up to 40% of the amino acids have their side chains chemically modified; and/or up to 20% of the amino acids have been deleted; provided that at least 50% of the amino acids in the parent sequence of (i) are maintained unaltered in the variant, and provided that the variant has autoimmune disease improving properties;

(iii) a sequence of any one of (i) or (ii) wherein at least one of the amino acids is replaced by the corresponding D- amino acid;

- (iv) a sequence of any one of (i) to (iii) wherein at least one of the peptidic backbones has been altered to a non-naturally occurring peptidic backbone;
- (v) a sequence being the sequence of any one of (i) to (iv) in reverse order; and
- (vi) a combination of two or more of the sequences of (i) to (v).

5 5. A method according to Claim 4 wherein the increase angiogenesis is for the treatment of a disease selected from; coronary artery diseases, peripheral artery diseases, endothelial vascular diseases, arteriosclerosis, various processes of wound and tissue healing such as healing of bone, tendon, endothelial lining (such as in ulcers in the stomach), for improving the success rates of cell transplantation techniques, as well as in
10 reconstructive surgery to help re-establish proper blood circulation to the reconstructed tissue.

6. A method according to Claim 1 for the decrease of angiogenesis wherein the sequence is selected from:

- (i) a sequence which is a continuous stretch of at least five amino acids present
15 in the native Tie-2 kinase in positions 904-923, a sequence which is continuous stretch of at least five amino acids present in the native c-kit kinase in positions 672-694, a sequence which is a continuous stretch of at least five amino acids present in the native VEGF kinase in positions 918-939 (alpha D region); a sequence a continuous stretch of at least five amino
20 acids present in the native GRK kinase in positions 383-406 (HJ-loop);
- (ii) a variant of a sequence according to any one of (i) wherein up to 40% of the amino acid of the native sequence have been replaced with a naturally or non-naturally occurring amino acid or with a peptidomimetic organic moiety; and/or up to 40% of the amino acids have their side chains
25 chemically modified; and/or up to 20% of the amino acids have been deleted; provided that at least 50% of the amino acids in the parent sequence of (i) are maintained unaltered in the variant, and provided that the variant has autoimmune disease improving properties;

- (iii) a sequence of any one of (i) or (ii) wherein at least one of the amino acids is replaced by the corresponding D- amino acid;
- (iv) a sequence of any one of (i) to (iii) wherein at least one of the peptidic backbones has been altered to a non-naturally occurring peptidic backbone;
- 5 (v) a sequence being the sequence of any one of (i) to (iv) in reverse order; and
- (vi) a combination of two or more of the sequences of (i) to (v).

7. A method according to claim 6 wherein the decrease of the angiogenesis is used for the treatment of a disease selected from: : cancer, aged-related macular degeneration (which are many times aggravated by normal neovascularization), diabetic retinopathy
10 (which are also caused by non normal neovascularization), rheumatoid arthritis, psoriasis, obesity, hemangioma (AIDS related), Kaposi sarcoma arteriosclerosis, and restenosis.

8. A method according to claim 7 wherein the cancer is solid tumor.

9. A method according to claim 8 wherein the solid tumor is selected from:
15 carcinoma, sarcoma, adenoma, hepatocellular carcinoma, hepatocellular carcinoma, hepatoblastoma, rhabdomyosarcoma, esophageal carcinoma, thyroid carcinoma, ganglioblastoma, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, synovioma, Ewing's tumor, leiomyosarcoma, rhabdotheliosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell
20 carcinoma, adenocarcinoma ,renal cell carcinoma, hematoma, bile duct carcinoma, melanoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, retinoblastoma,, multiple myeloma, rectal carcinoma, thyroid
25 cancer, head and neck cancer, brain cancer, cancer of the peripherals nervous system, cancer of the central nervous system, neuroblastoma, cancer of the endometrium, as well as metastasis of all the above.

10. A method according to claim 1 wherein the compound comprises a sequence depicted in Fig. 1

11. A method according to Claim 1, wherein the compound is linear.
12. A method according to Claim 11, wherein the compound comprises a hydrophobic moiety at one of its terminals.
13. A method according to Claim 12, wherein the hydrophobic moiety is a
5 hydrocarbon having 4 to 20 carbon atoms.
14. A method according to Claim 12, wherein the compound comprises the hydrophobic moiety conjugated to the N-terminal of any one of the sequences as defined in Claim 1(a) to 1(i).
15. A method according to Claim 1, wherein the compound is a hydrophobic moiety
10 conjugated to the N-terminal of any one of the sequences as defined in Claim 1(a) to 1(i).
16. A method according to Claim 11, wherein the compound comprises a hydrophobic moiety conjugated to Gly, present at the N-terminal of any one of the sequences as defined in Claim 1(a) to 1(i).

Fig. 1

Peptide	Origin	Nomenclature	Comments	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	AP Result	AMC	HFG	B16	Melanogenesis Results	Sponge Assay	Eye Assay		
K024H107	HJ	Acetyl	Acetylated	L	L	R	R*	H	S																inhibition				stimulation				
K024H112	HJ	Myristyl-G	Myristylated	L	L	R	R*	H	S	I															inhibition				stimulation				
K024H124	HJ	Lauroyl-G	Lauroylated	L	L	R	R*	H	S	I															inhibition				stimulation				
K063D103	aD	Myristyl-G	Myristylated	N	L	L	D	F	L	R	K	S	R	V	L										inhibition	inhibition							
K063D104	aD	Myristyl-G	Myristylated	I	E	Y	A	P	H	G	N	L	L	D	F	L									inhibition								
K063D105	aD	Myristyl-G	Myristylated	N	L	L	D	F	L	R	K	S	R	V	L										inhibition of SIP, VEGF and SCF stimulation effects				stimulation	inhibition			
K063D110	aD	Myristyl-G	Myristylated	N	L	L	N	F	L	R	K	S	R	V	L										inhibition			inhibition					
K063D902	aD	Stearyl-G	Stearylated	N	L	L	D	F	L	R	K	S	R	V	L											inhibition	inhibition						
K063D903	aD	Stearyl-G	Stearylated	N	L	L	D	F	L	R	K	S	R																				
K063D904	aD	Stearyl-G	Stearylated	L	D	F	L	R	K	S	R	V	L																				
K063D905	aD	Stearyl-G	Stearylated	L	D	F	L	R	K	S	R	V	L	E	T												inhibition	inhibition					
K063D906	aD	Stearyl-G	Stearylated	H	G	N	L	L	D	F	L	R	K	S	R																		
K063D908	aD	Stearyl-G	Stearylated	G	N	F	L	R	K	S	R	V	L	Q												inhibition	inhibition						
K068D001	aD	Acetyl		G	N	L	S	N	F	L	R	A	K	R	N	L	F	V	P							inhibition			inhibition				
K068D002	aD	Acetyl		G	N	L	S	N	F	L	R	A	K	R	N	L	F	V	P	K\$													
K068D101	aD	Myristyl-G	Myristylated	G	N	L	S	N	F	L	R	A	K	R	N	L	F	V	P							inhibition	stimulation	inhibition	inhibition				

Fig. 1 (Continued)

Peptide	Origin	N-Terminus	Comments	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	AP Results	AMS	HEC	B16	Melanogenesis Results	Sponge Assay	Eye Assay	
K068D102	aD	Myristyl-G	Myristylated	V	E	F	S	K	F	G	N	L	S	N	F	L	R	A	K	R	N	L	P	V	inhibition, abrogation of VEGF and S1P effect	stimulation	stimulation	inhibition	NE		
K068D901	aD	Stearyl-G	Stearylated	R	F	R	Q	G	K	D	Y	V	G	E	L									stimulation		inhibition					
K068D902	aD	Stearyl-G	Stearylated	N	F	L	R	A	K	R	N	L	F											inhibition							
K068D903	aD	Stearyl-G	Stearylated	N	F	L	R	A	K	R	N	L	F	V	P									inhibition							
K068D904	aD	Stearyl-G	Stearylated	T	Y	L	R	S	K	R	N	Q	F	V	P									inhibition							
K068D905	aD	Stearyl-G	Stearylated	T	Y	L	R	S	K	R	N	Q	F											inhibition							
K068D906	aD	Stearyl-G	Stearylated	T	Y	L	R	A	K	R	N	Q	F	V	P									inhibition slight							
K068D909	aD	Stearyl-G	Stearylated	F	R	G	K	N	Y	L	N	Q	L											stimulation		inhibition					
K107H101	HI	Myristyl-G	Myristylated																					stimulation						vessels stabilisation	
K147D101	aD	Myristyl-G	Myristylated	D	L	L	N	F	L	R	R	K	R	D	S	P								stimulation			stimulation				
K147D101 + SCF																								stimulation							
K147D102	aD	Myristyl-G	Myristylated	N	L	L	N	F	L	R	R	K	R	N	S	P								inhibition				stimulation			
K147D102 + SCF																								inhibition							
K147D103	aD	Myristyl-G	Myristylated	N	L	L	N	F	L	R	R	K												inhibition			inhibition	stimulation			

Fig. 1 (Continued)

Peptide	Origin	N-terminal	Comments	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	AR Results	A19	SMG	HEC	B16	Melanogenesis Results	Spont. Assay	Eye Assay
K147D203	aD	Decanoyl-G		N	L	L	N	F	L	R	R	K													inhibition					NE		
K147D302	aD	Myristyl-G	Myristylated	G	N	L	L	N	F	L	R	R	K	R											inhibition (low)					stimulation		
K147D305	aD	Myristyl-G	Myristylated	G	N	L	L	N	F	L	R	R	K																stimulation			
K147D306	aD	Myristyl-G	Myristylated	G	N	F	L	R	R	K	R																		stimulation			
K147D903	aD	Stearyl-G	Stearyl-G	N	L	L	N	F	L	R	R	K													inhibition	inhibition				NE		
K147D903 + SCF																									inhibition	inhibition				stimulation		
K147D904	aD	Stearyl-G	Stearyl-G	N	F	L	R	R	K	R	N	S	F												inhibition							
K147H103	HJ	Myristyl-G	Myristylated	G	S	S	P	Y	P	G	M	P																		stimulation		

Fig. 2A

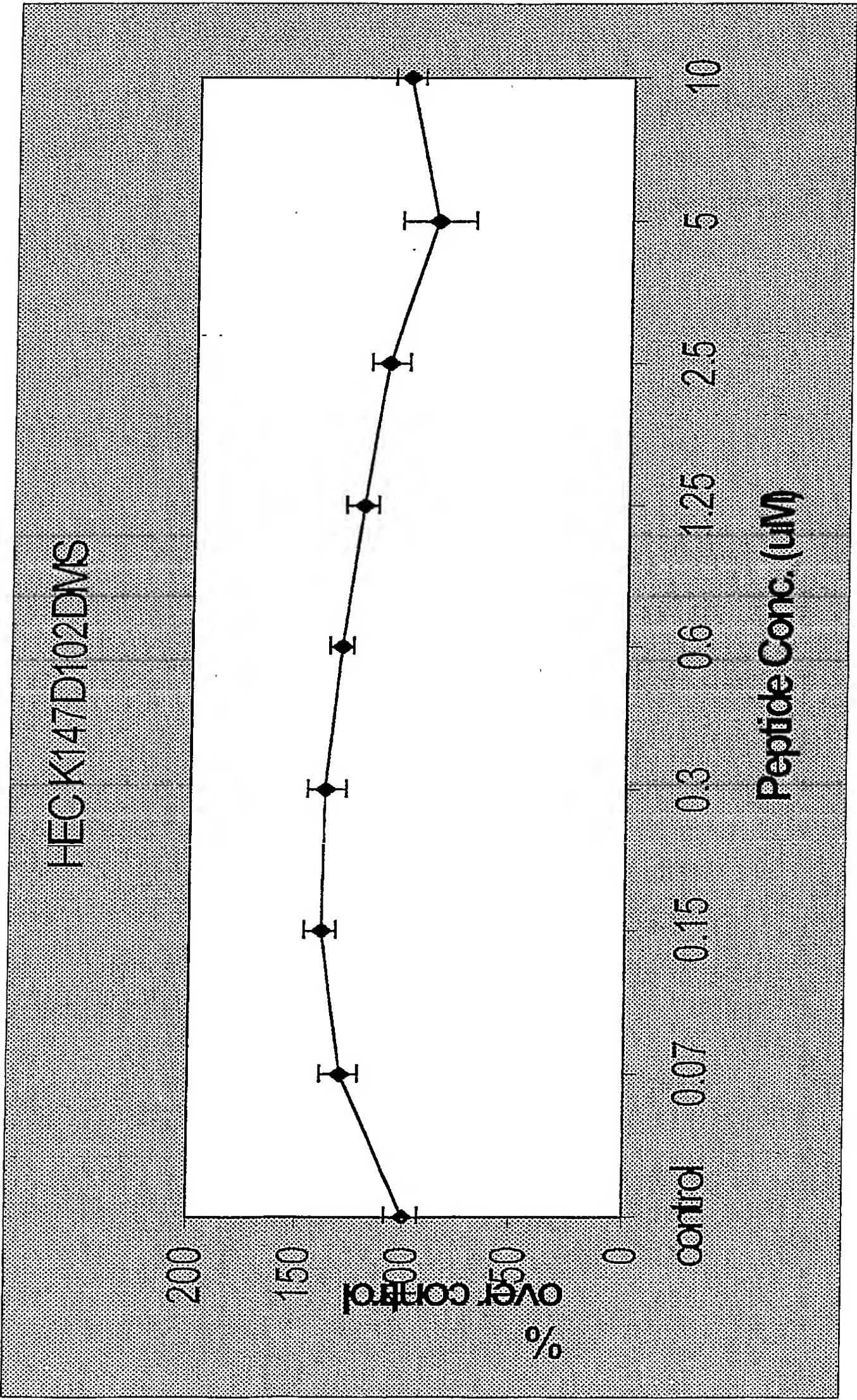


Fig. 2A (continued)

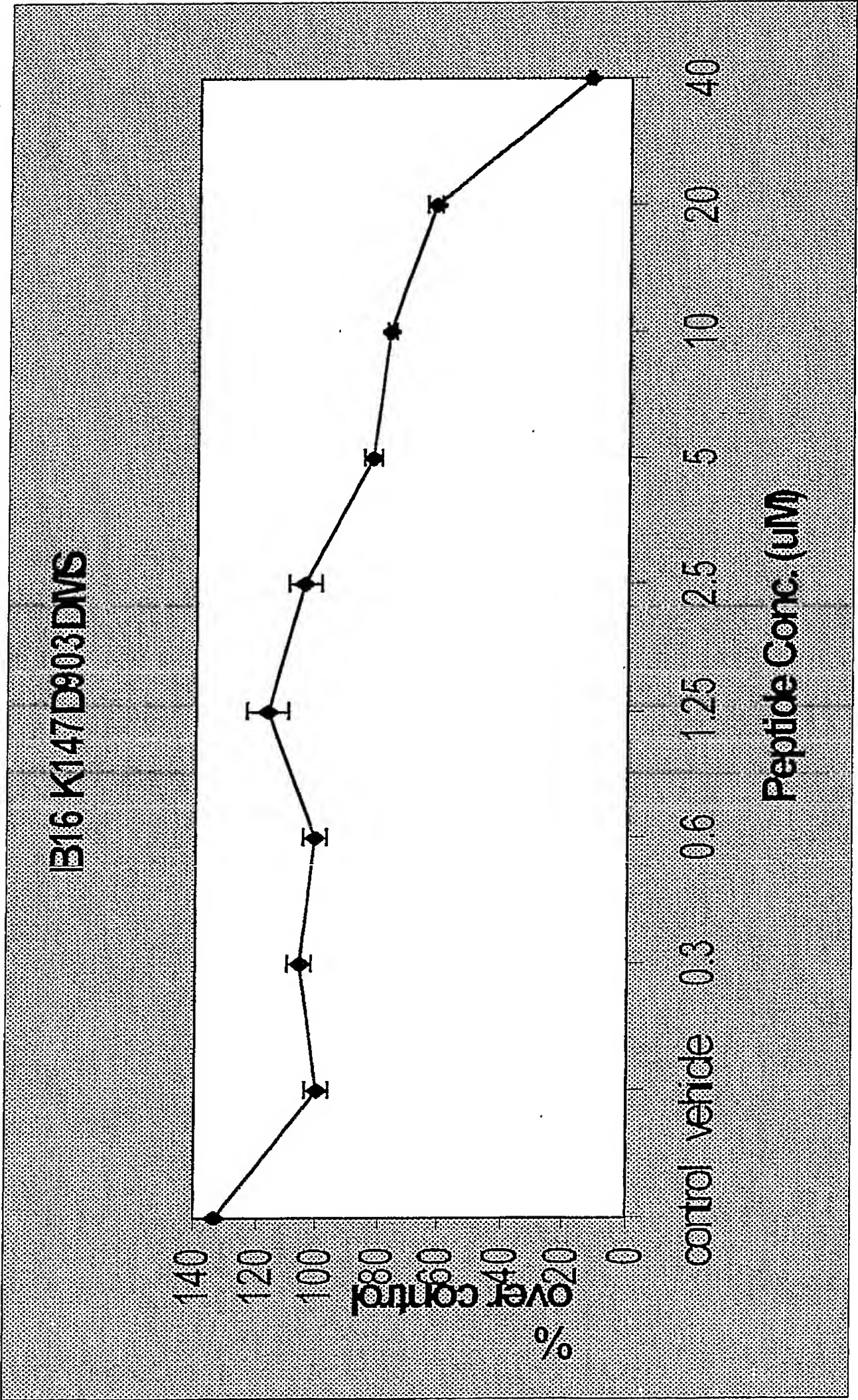


Fig. 2A (continued)

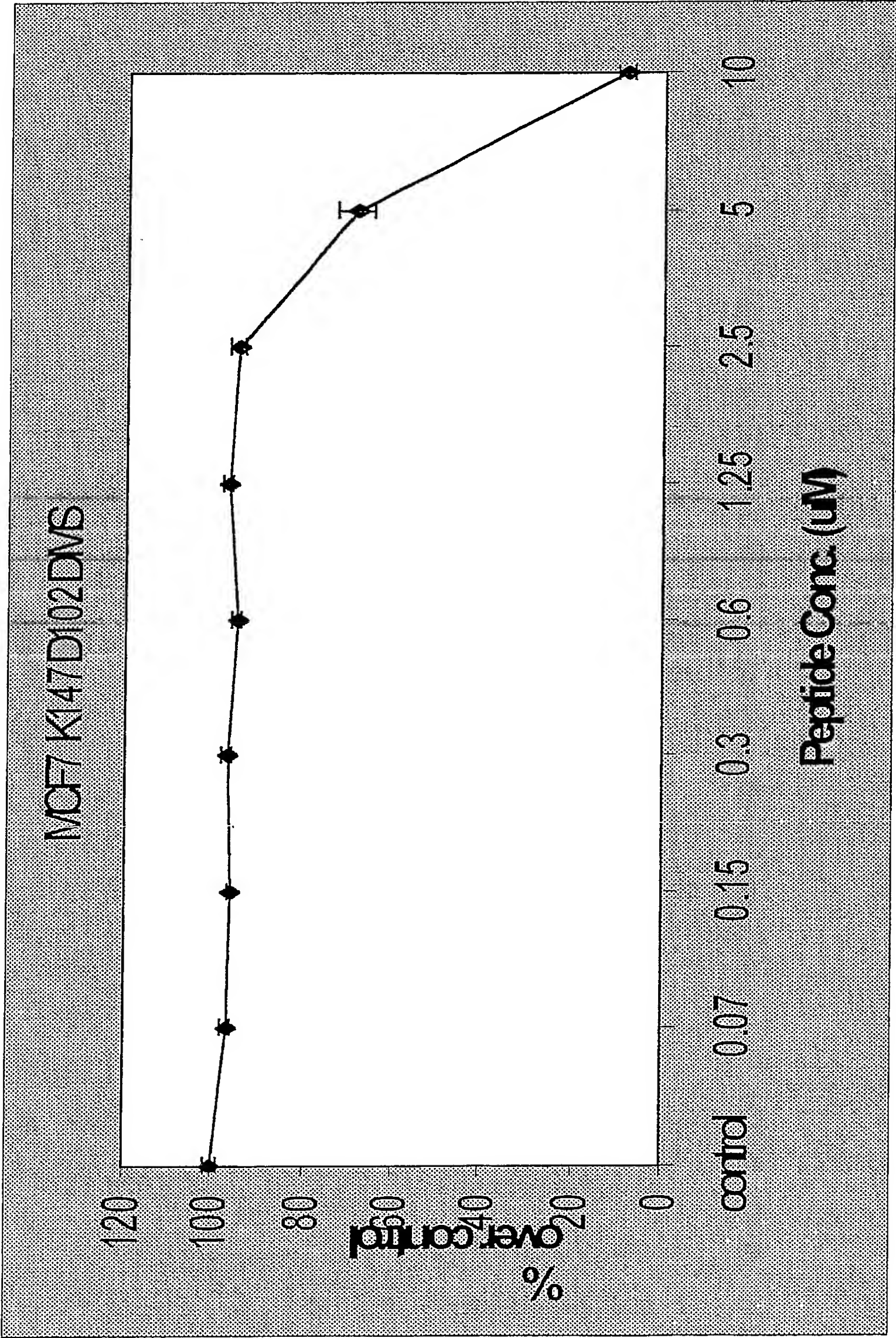


Fig. 2A (continued)

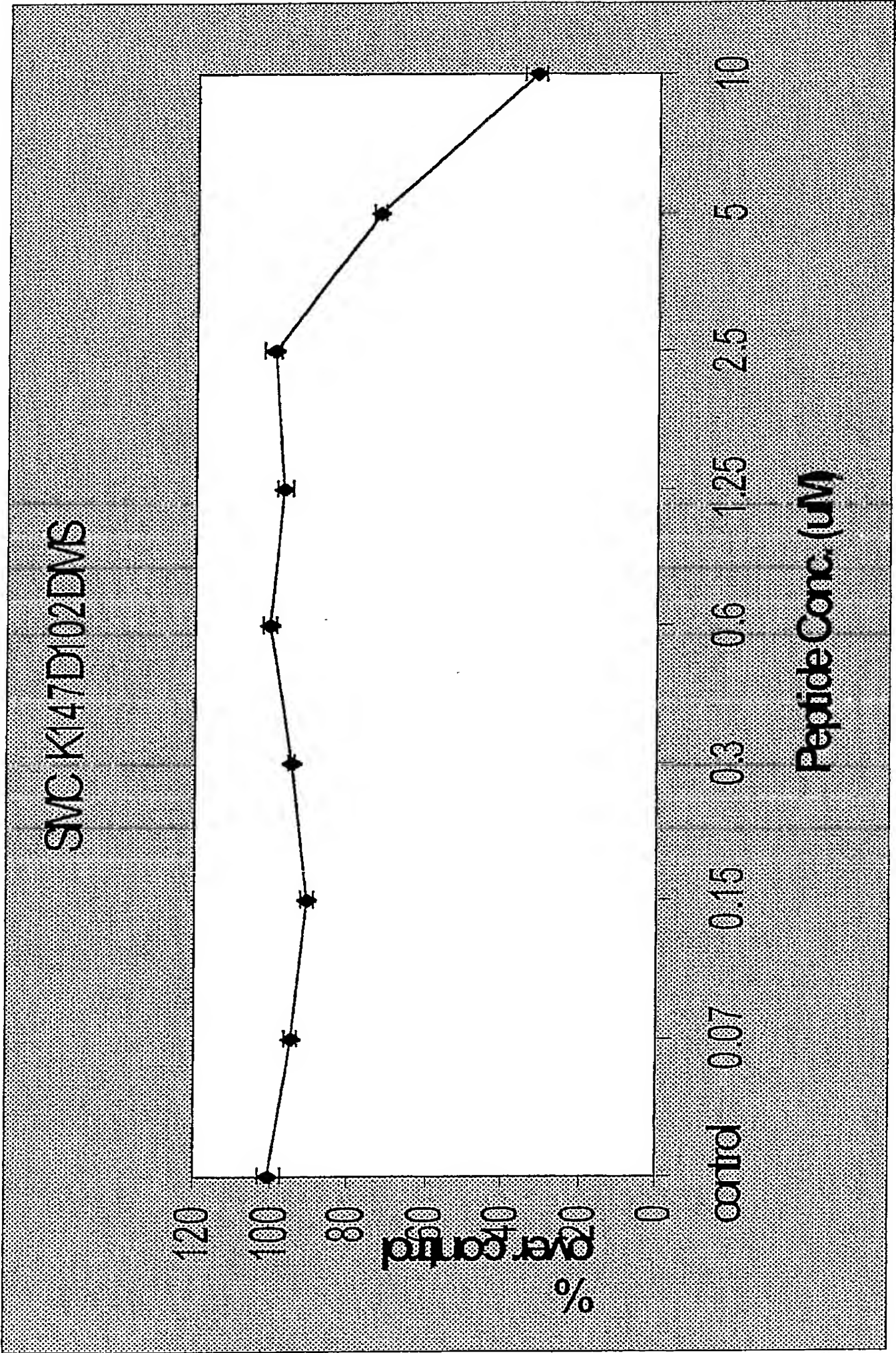


Fig. 2B

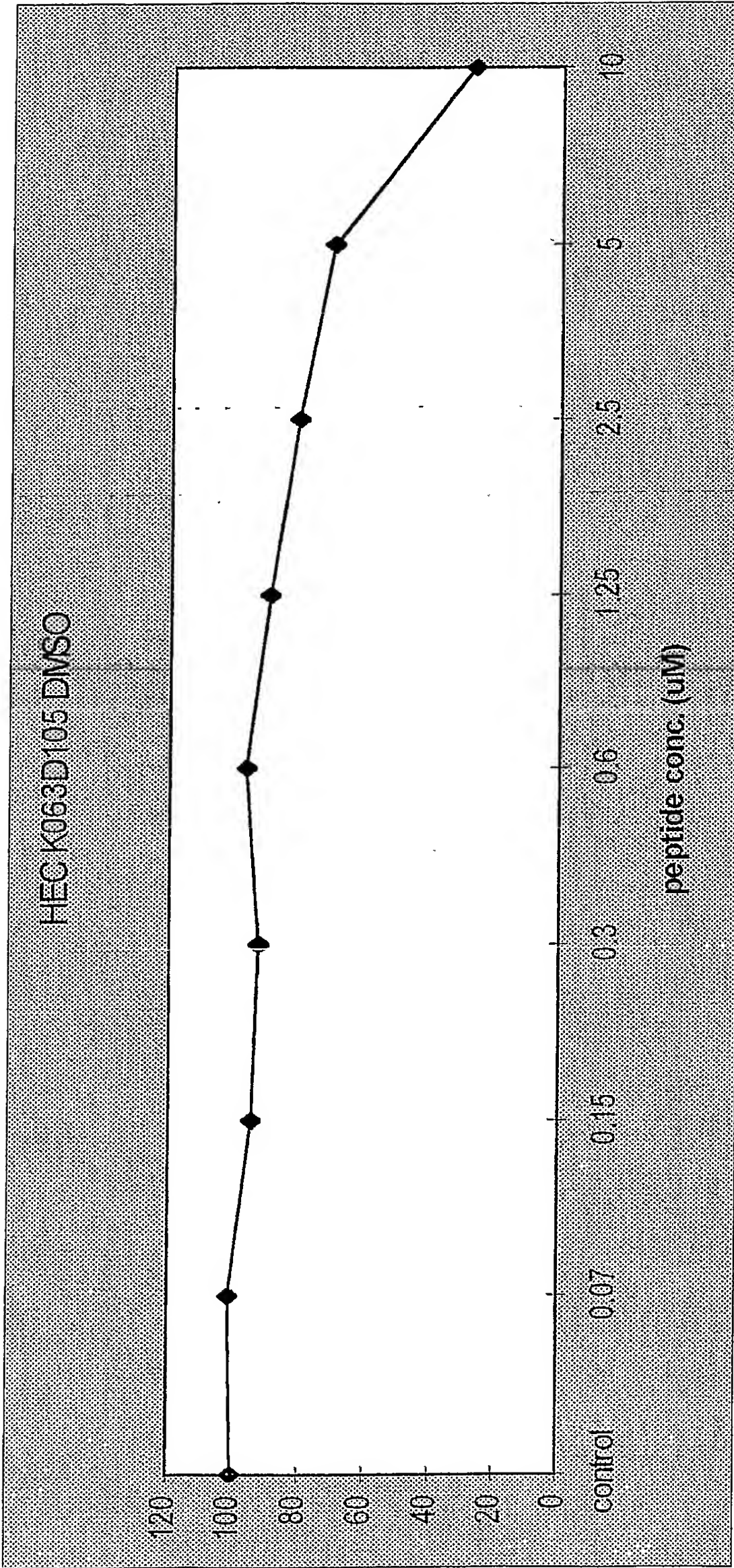


Fig. 2B (continued)

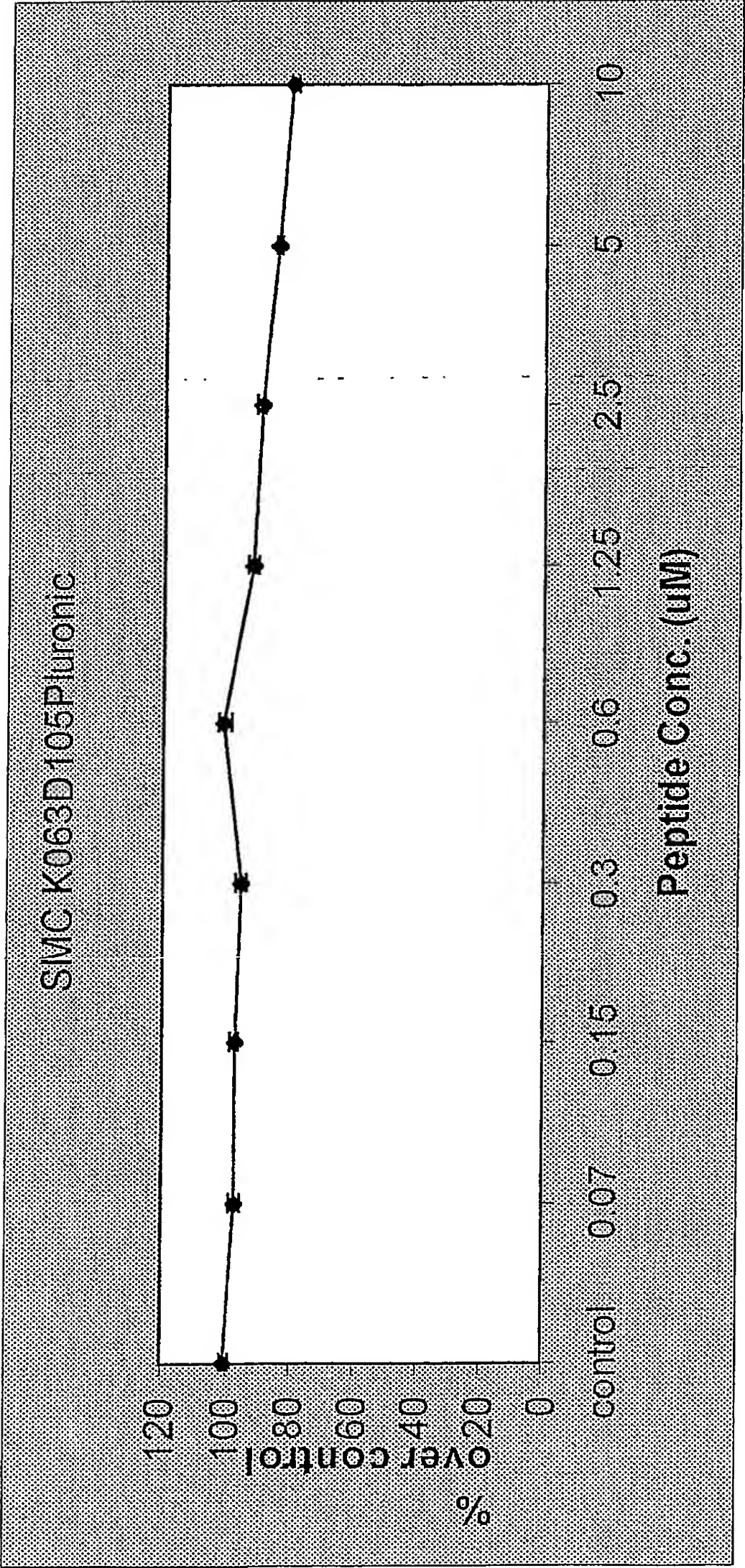


Fig. 2C

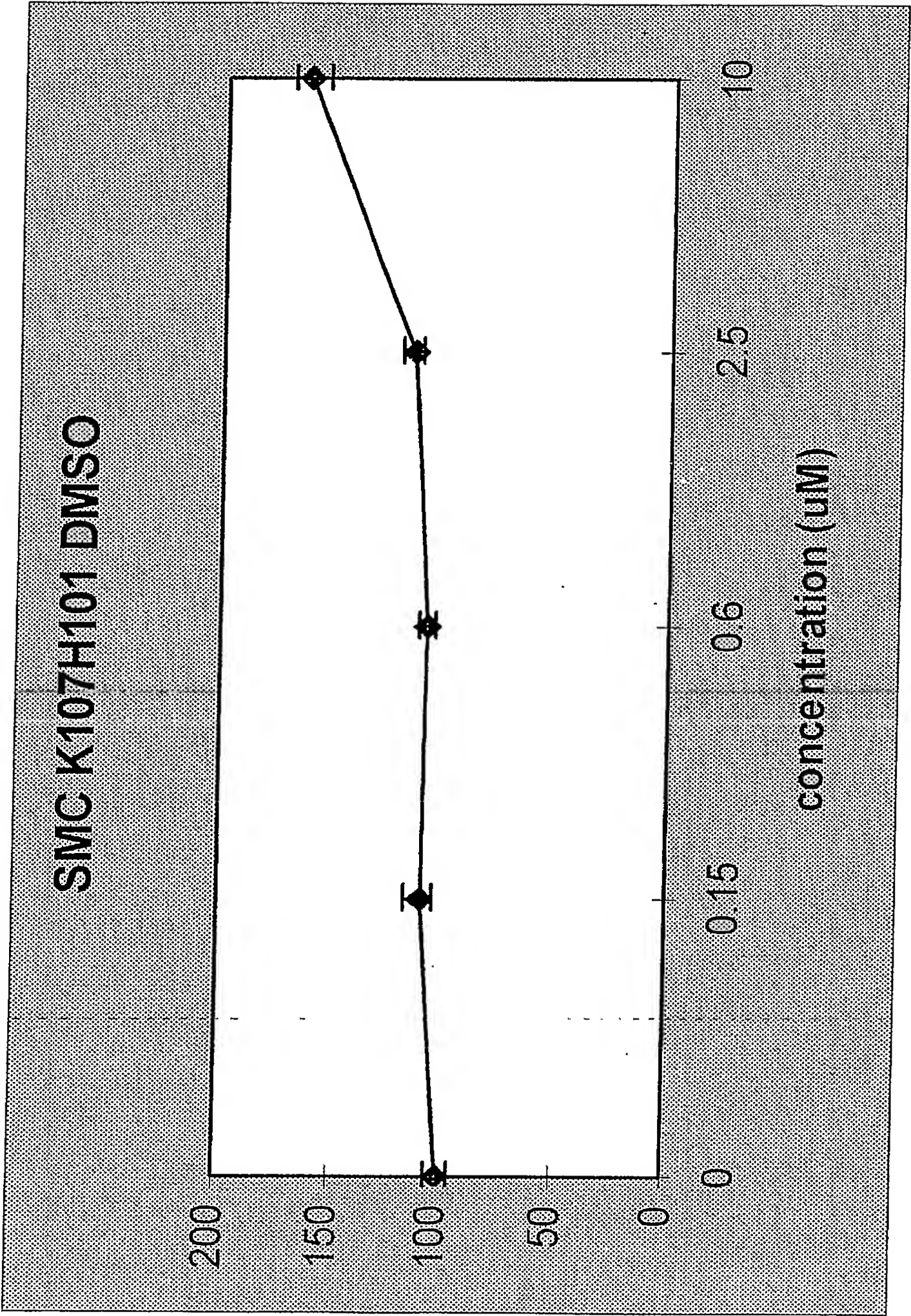


Fig. 2D

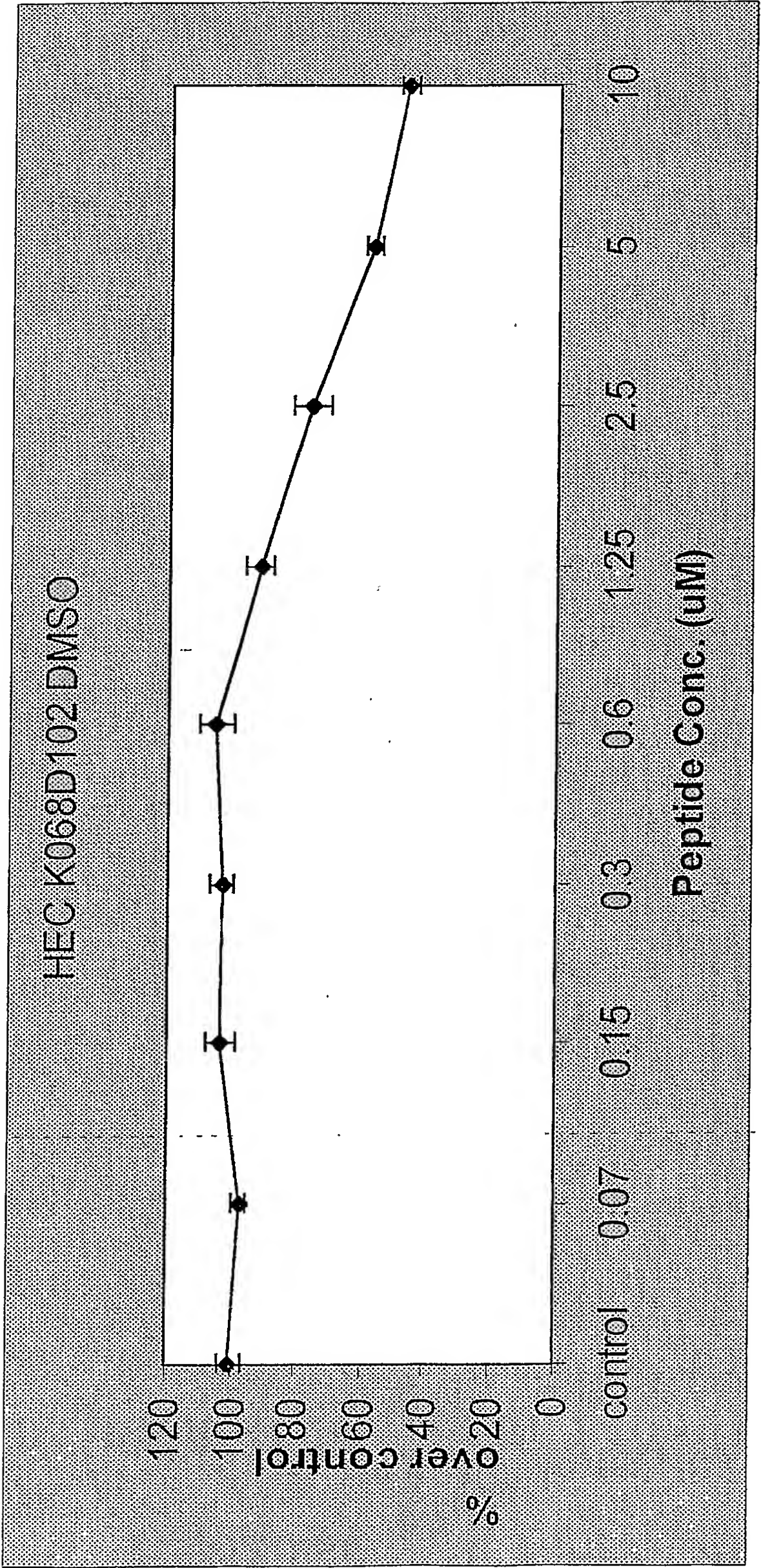


Fig. 2D (continued)

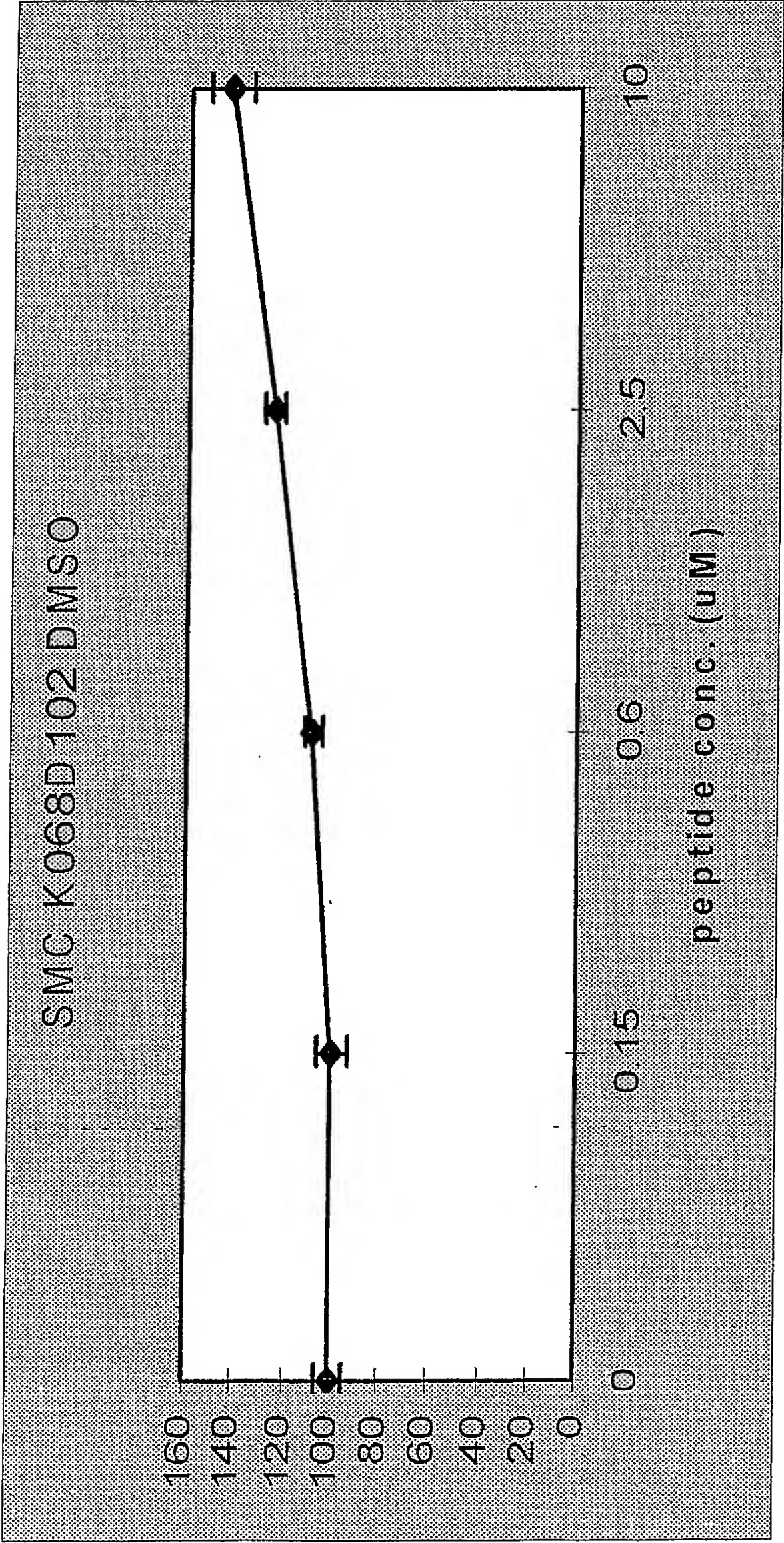


Fig 3A

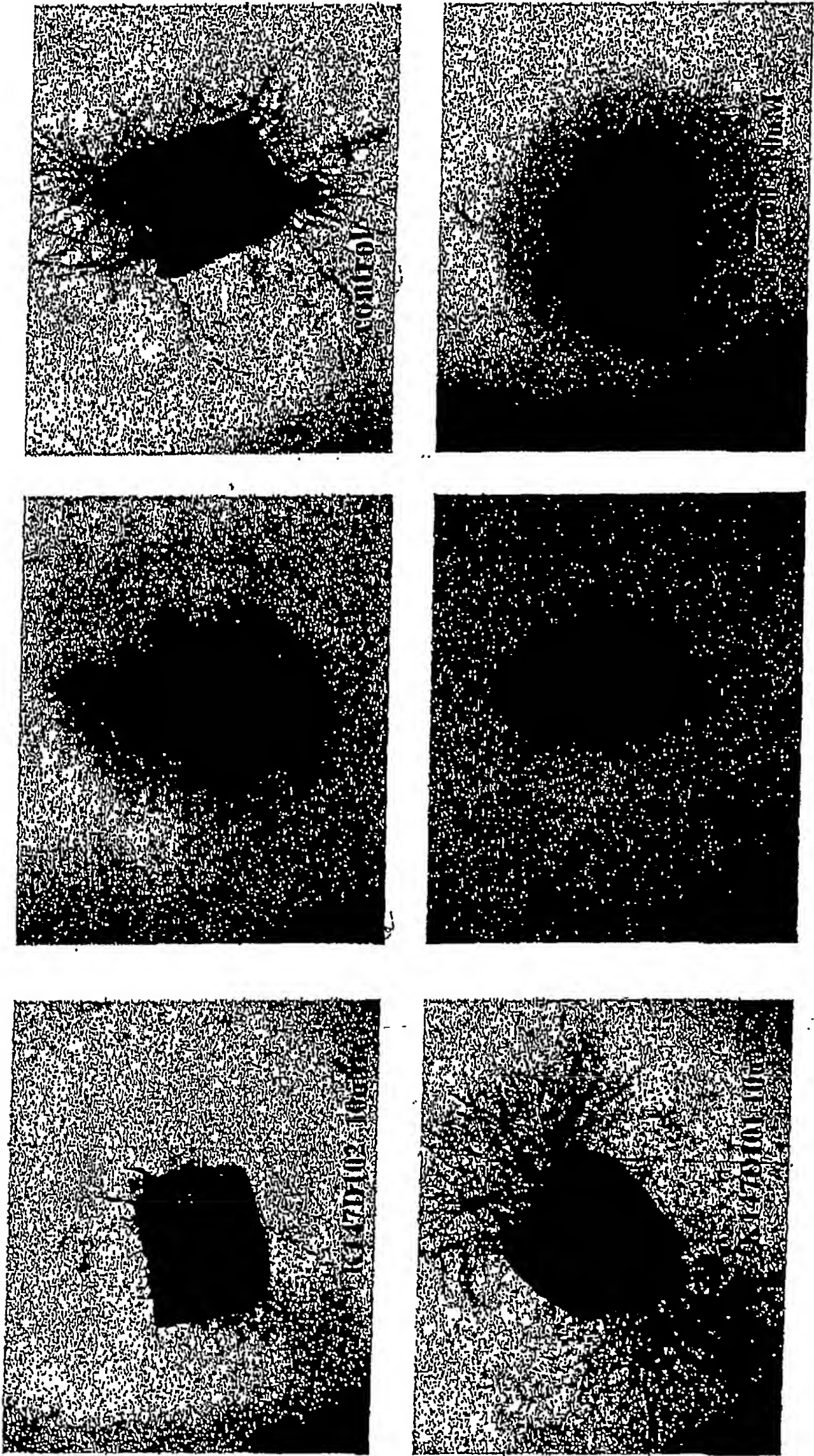


Fig. 3B

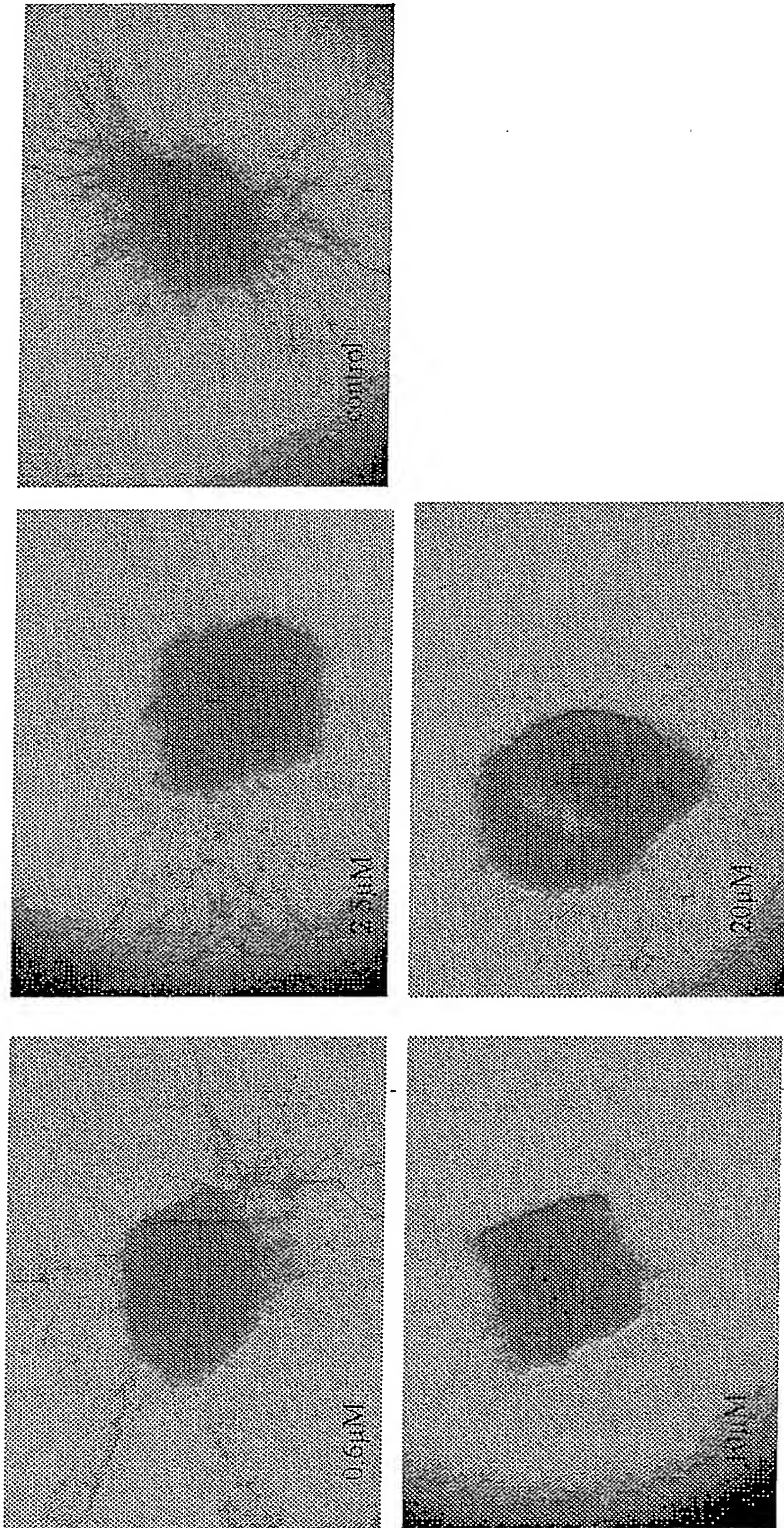


Fig. 3C

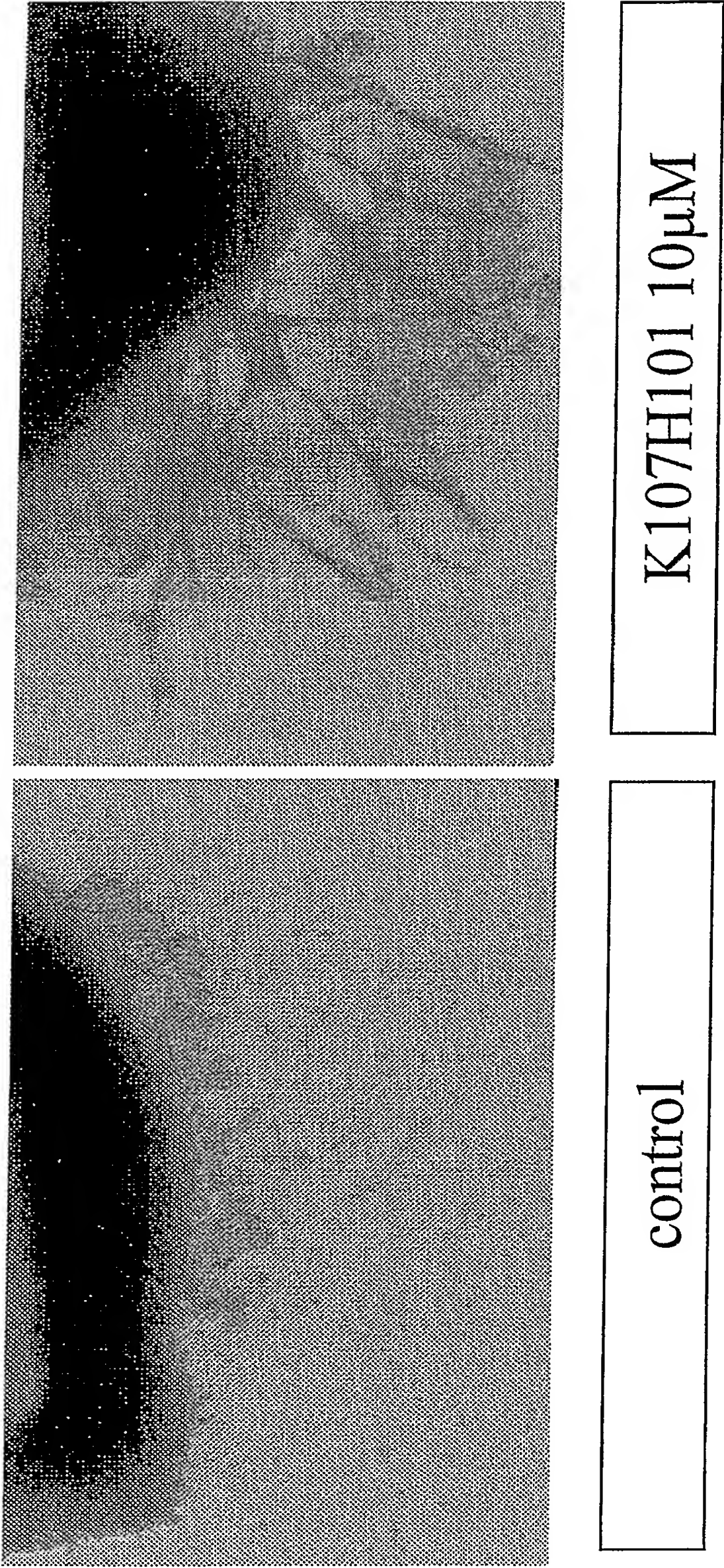
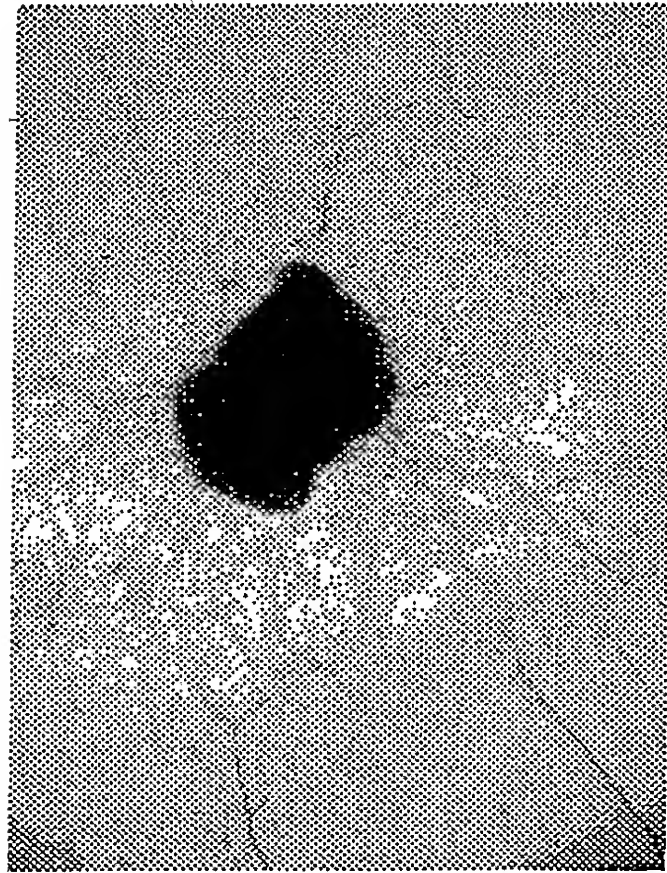
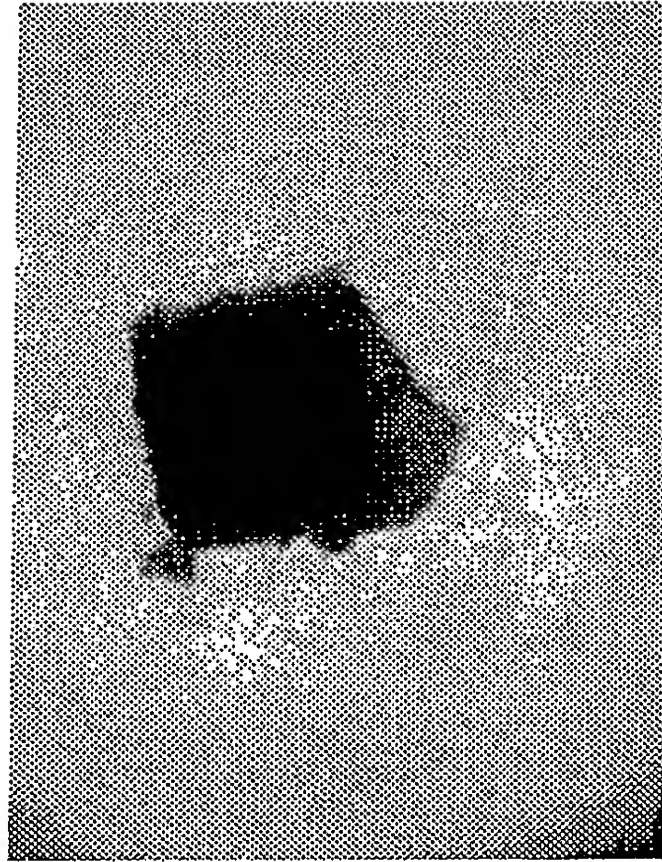


Fig. 3D



VEGF 25ng/ml



VEGF+K068D102

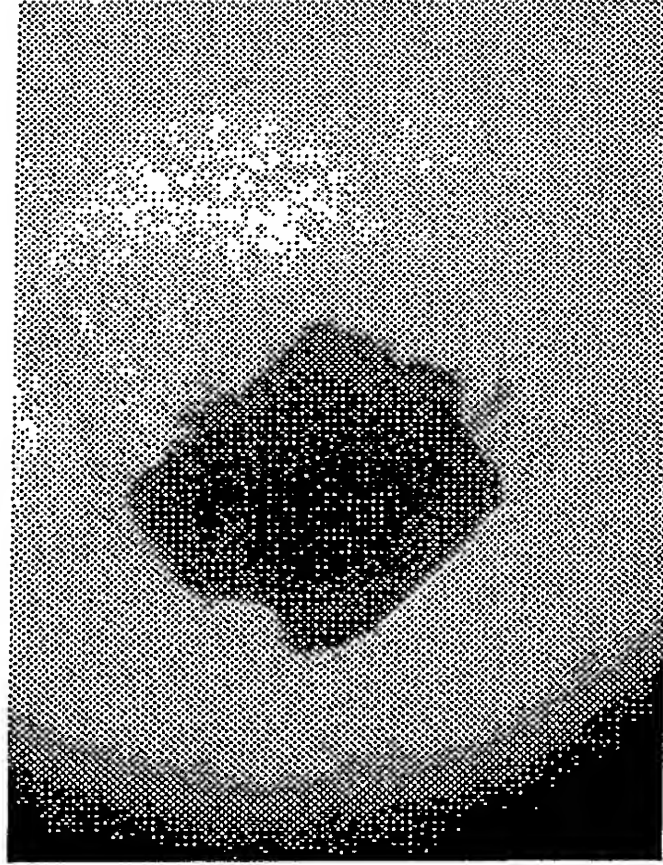
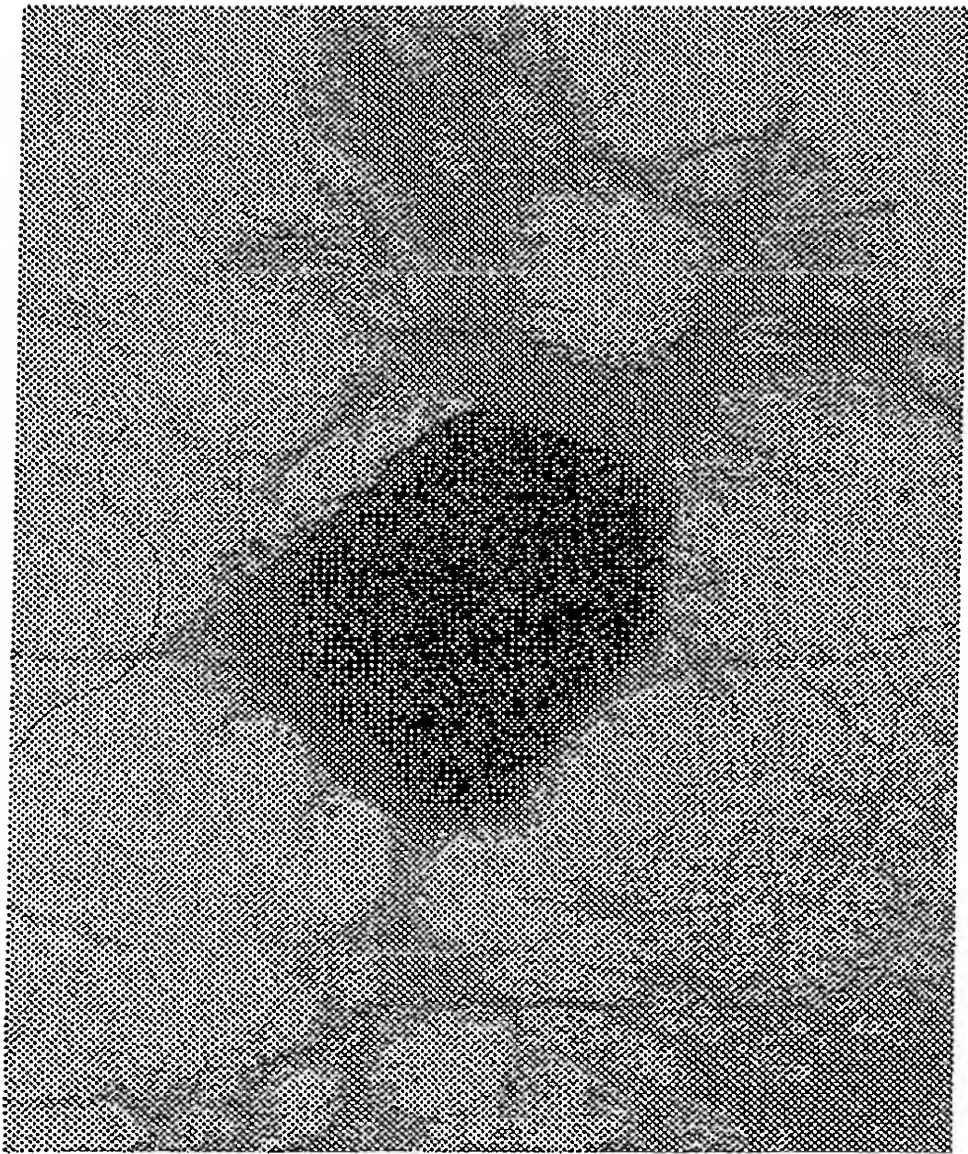
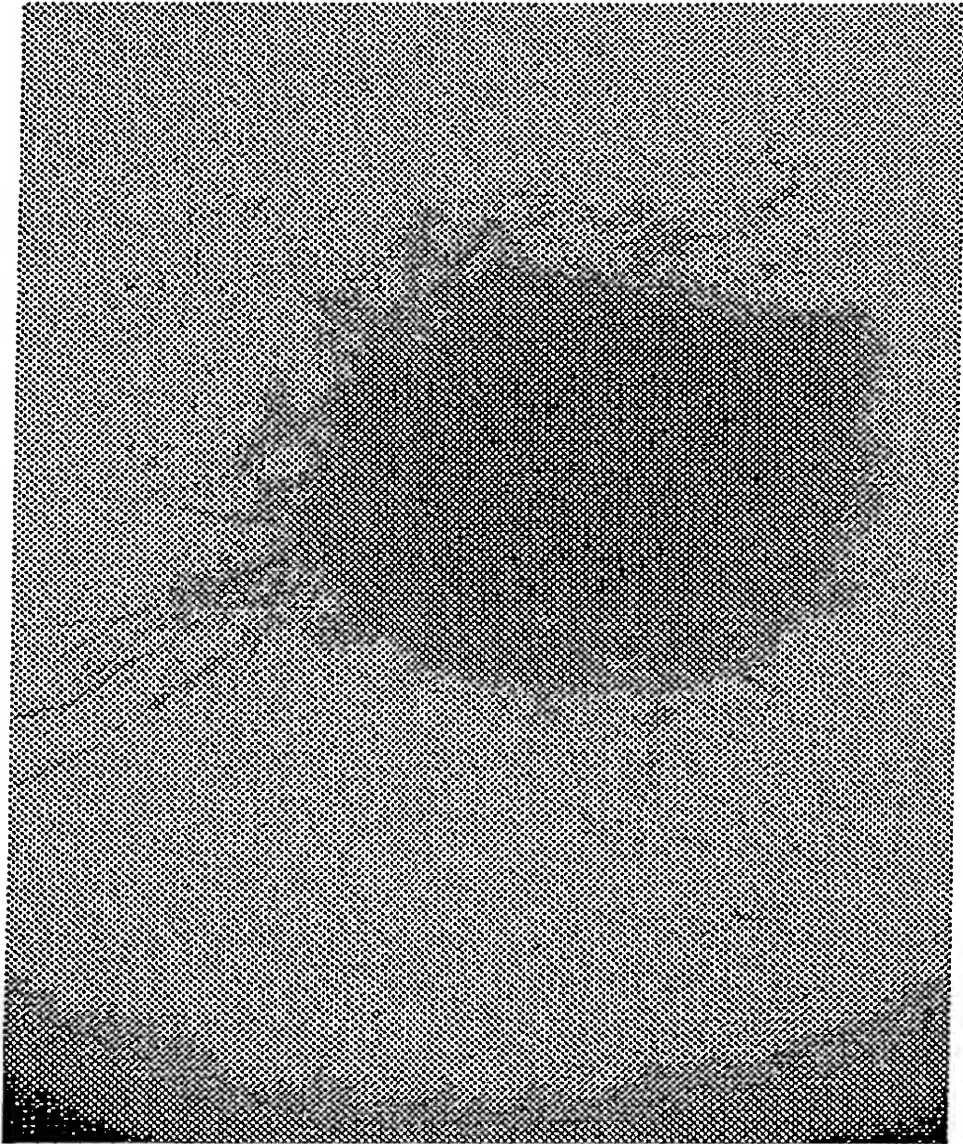
K068D102 10 μ M

Fig. 3E



control



K024H124 10μM

Fig. 4

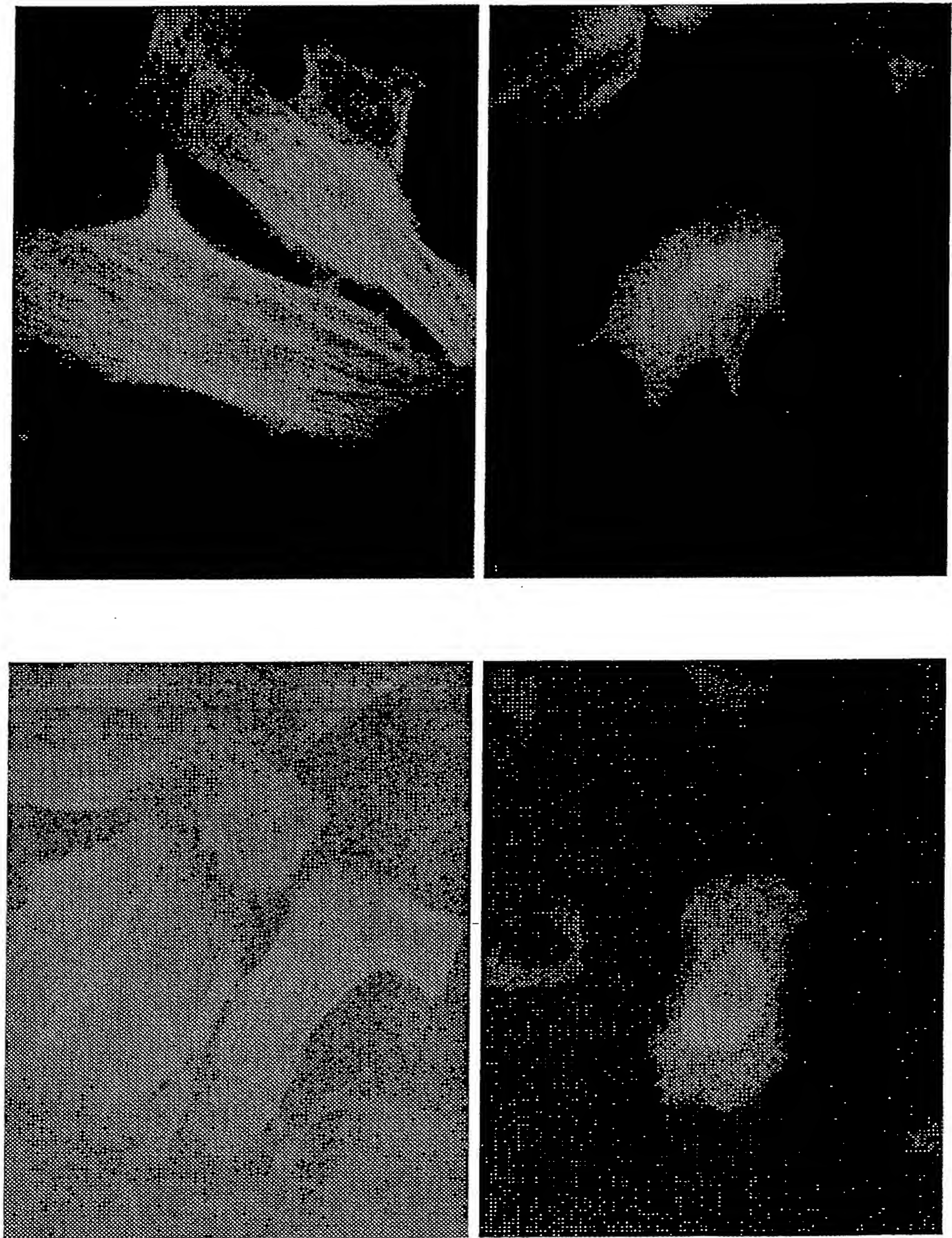


Fig. 5

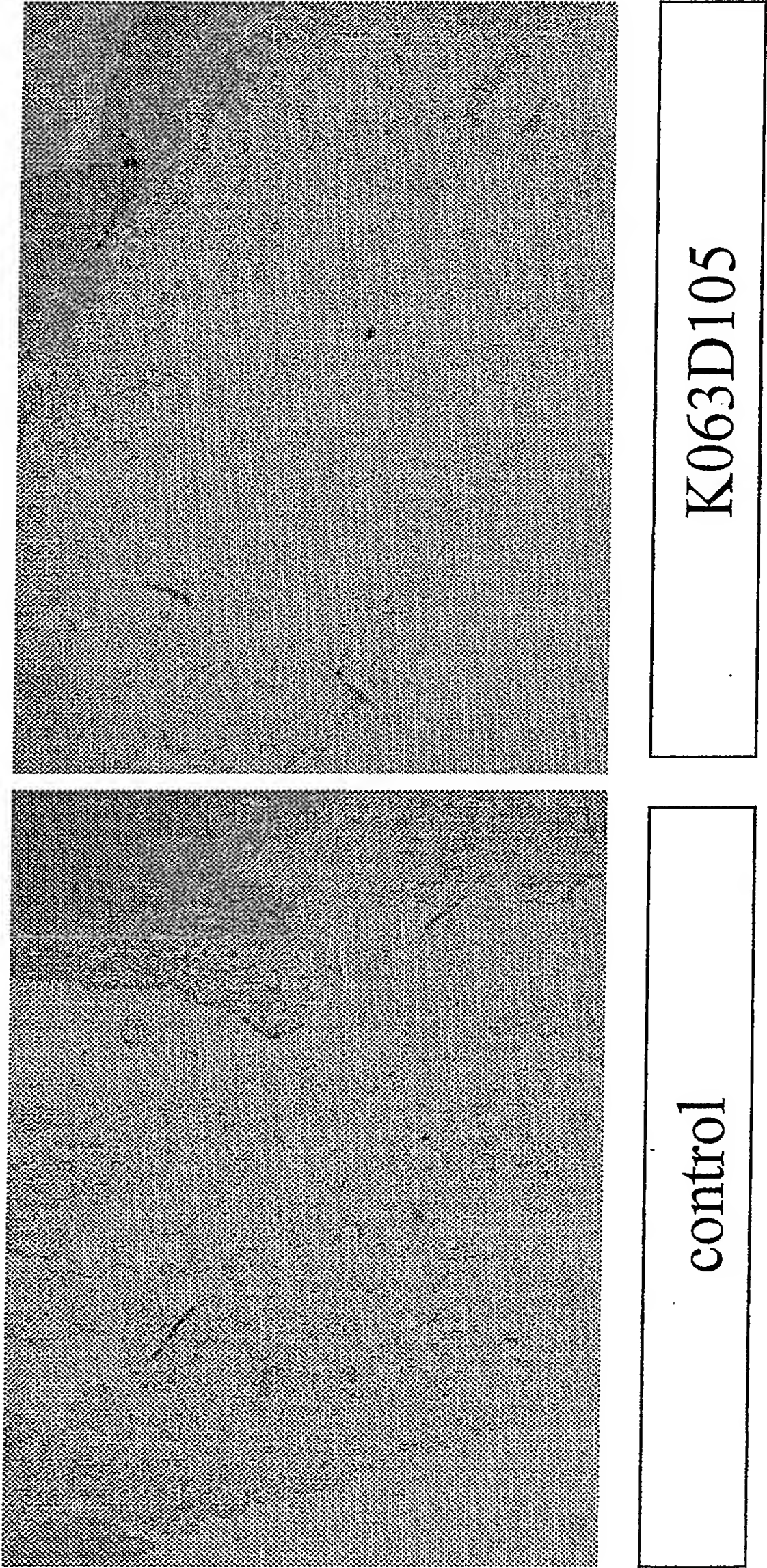


Fig. 6A

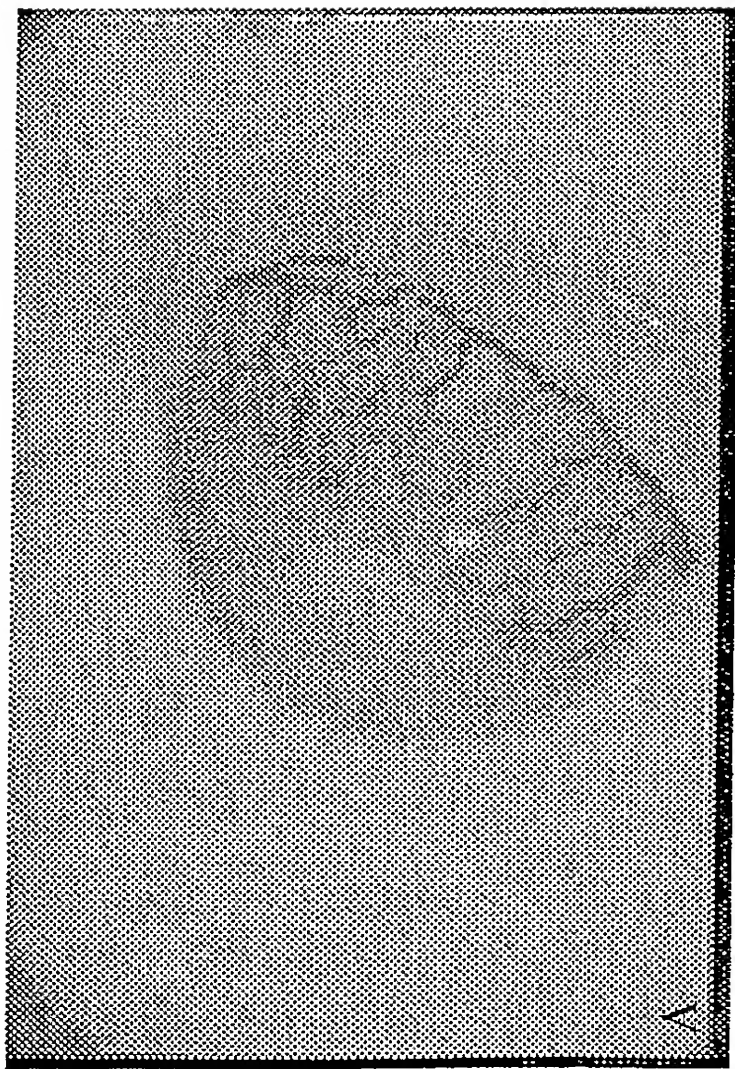


Fig. 6B

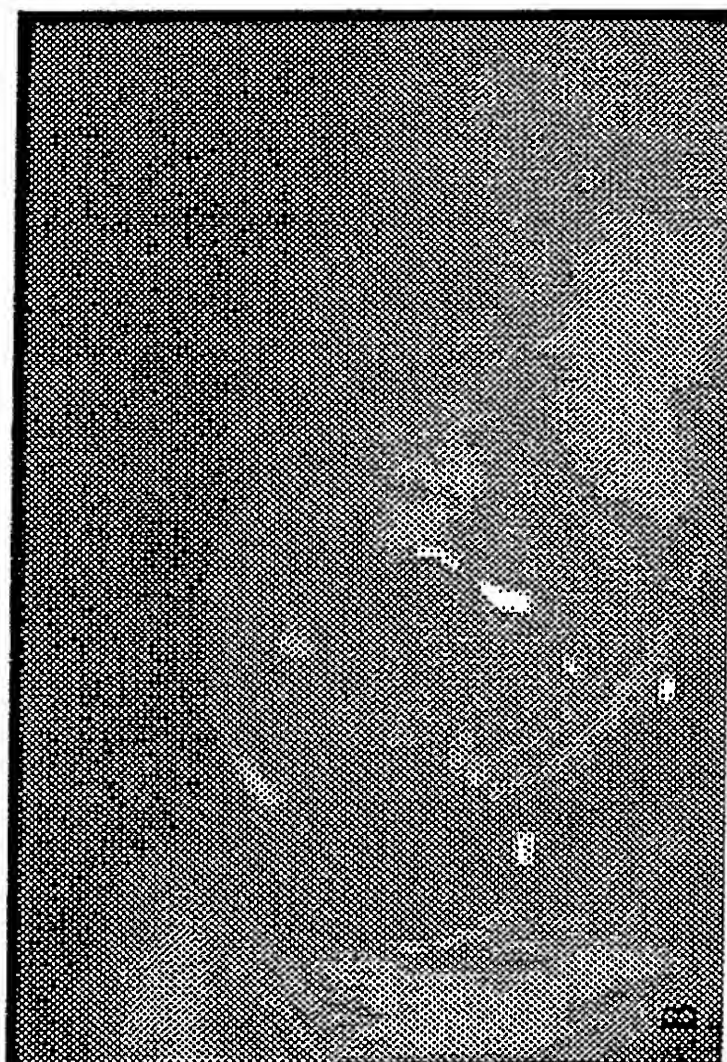


Fig. 6C

